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Complementary action of Gibberellic acid and auxins in Pea internode extension

BY

P. W. BRIAN AND H. G. HEMMING

(*Akers Research Laboratories, Imperial Chemical Industries Ltd., Welwyn, England*)

With five figures in the text

ABSTRACT

Gibberellic acid (GA) induced extension of green pea-stem sections in light only if an auxin was also present. Of the auxins tested 3-indolylacetic acid, 2-methyl-4-chloro-phenoxyacetic acid, 2:4-dichloro-phenoxyacetic acid and 1-naphthylacetic acid were effective in increasing extension of sections and in eliciting a response to GA. Excised internodes from plants pre-treated with GA extended appreciably faster *in vitro* than those from untreated plants only if an auxin was supplied in the incubation medium. This and other evidence suggests that in the intact plant GA elicits a growth-response only in the presence of auxin. By comparing growth-rates of excised internodes *in vitro* and of intact internodes *in vivo* under comparable conditions, using untreated plants and plants pre-treated with GA, evidence has been obtained that in untreated plants growth-rate is somehow limited to a level below that made potentially possible by the endogenous auxin supply; treatment with GA appears to release the plant from this state of inhibition. Growth of intact pea internodes is considered to be regulated by a three-factor system, consisting of auxin, an inhibitory system, and a hormone with physiological properties similar to those of GA.

INTRODUCTION

WE have shown in a previous paper (Brian, Hemming, and Radley, 1955) that though gibberellic acid (GA) has auxin-like activity in so far as it induces cell elongation in shoot tissues, in many other respects it differs from the auxins. It was not considered possible, on the evidence then available, to decide whether gibberellic acid should be regarded as an auxin or not. We return to this point in the present paper; the aim of the investigation now reported has been to determine whether GA is auxin-like in its physiological activity, or, if not, whether GA and auxins act independently or in association. This could not be satisfactorily achieved using intact plants, since effective control of the natural auxin supply is difficult to achieve in so complex a system. We therefore decided to use tissue sections as our main experimental material. It is doubtful how far growth of fragments of etiolated tissue is representative of normal growth; accordingly we have used sections cut from growing internodes of light-grown plants of *Pisum*, measuring their extension in light by the technique of Galston and Baker (1951). However, whereas Galston and Baker used sections from a tall variety,

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Alaska, we have used sections from a dwarf variety, Meteor, because intact plants of dwarf peas show a much greater response to GA than tall varieties (Brian and Hemming, 1955). It was hoped that results obtained by this *in vitro* technique could reasonably be used to explain the behaviour of GA in the intact plant.

MATERIALS AND METHODS

The technique finally used differed from that of Galston and Baker in several respects, as indicated below.

Plant material. Meteor pea seedlings were grown in a greenhouse, unheated in summer but heated in winter to *c.* 15° C. Supplementary light from Daylight fluorescent tubes was provided in winter. Sections were cut from the 5th or 6th internode, the internode used being in each case the uppermost visible internode. The sections were 5·1 mm. in length, cut with a specially designed guillotine. It was only possible to obtain one section from each plant since the internodes of the variety Meteor are very short. Galston and Baker used plants grown in controlled environment chambers; our use of greenhouse-grown plants, where environmental control was far from adequate, was undoubtedly a source of experiment to experiment variation but this was not unduly serious, as will be seen from the results presented.

Incubation and measurement of sections. The solutions in which the sections were to be grown were dispensed in 0·5 ml. quantities in agglutination trays. These are made of 'Perspex' sheet, and each has 80 hemispherical depressed cells of 16 mm. diameter and 10 mm. maximum depth. The plants were drawn at random from the greenhouse bench and sections placed in the solution as soon as cut, one section to each cell. For each treatment in any experiment 30–40 replicate sections were used; during the process of section cutting the sections were assigned ten at a time to each treatment in random order, and when sections were measured at the end of the experiment they were measured in the same order, so that all had equal incubation periods. The trays of sections were placed in closed 'Perspex' humidity chambers at 15° C. in a controlled environment chamber and illuminated at 800 f.c. with light from a mixture of mercury-vapour, tungsten-filament, and neon lamps. After 24 hours' incubation the sections were measured to 0·1 mm. using a Hilger Inspection Enlarger, which projects an enlarged (× 10) image of the section. In many experiments the sections were also measured initially but it was found that this was unnecessary, since the guillotine produced sections of very even length.

In earlier experiments the sections were pooled after cutting in shallow vessels of distilled water and subsequently placed in the test solution. However, it was found that sections which received this treatment, which was in effect an inefficient washing (partially washed sections), grew less vigorously than those placed immediately in the test solution (unwashed sections) unless both indolylacetic acid (IAA) and sucrose were supplied, and results were somewhat more variable between experiments (see Table I). Extension of the sections also commenced in the distilled water. Since sections remained in the distilled water for unequal times the amount of washing and of preliminary extension was not equal for all sections; this probably accounted for the greater variability of results obtained with partially washed sections. Hence in all experiments described in this paper unwashed sections were used unless it is stated to the contrary. All solutions for test were made up at pH 6·1 in M/60 phosphate buffer. Some experiments with intact plants have been carried out for comparative purposes; the methods used are described in the text.

TABLE I

Mean Extension (mm.) of Meteor Pea Sections in Various Combinations of Indolylacetic Acid (IAA), Buffer and Sucrose

(All data are means from 10–12 experiments; the standard deviations refer to variation between experiments)

Pre-treatment of sections	Buffer only	2% sucrose	10 μg./ml. IAA	IAA+ sucrose
Partially washed sections .	0·9±0·32	1·1±0·31	2·1±0·65	3·4±0·58
Unwashed sections . .	1·4±0·29	1·5±0·32	3·3±0·54	3·7±0·44

RESULTS

Response of sections to sucrose and auxins

Galston and Baker (1951) found that sucrose induced no extra growth in light. We found that 2 per cent. sucrose consistently induced increased growth in the presence of optimal or near-optimal concentrations of IAA or other auxins, though not usually in their absence (e.g. Tables I and II). Higher concentrations than 2 per cent. were sometimes inhibitory. In order to ensure that sucrose deficiency was not limiting growth, 2 per cent. sucrose was included in the test solutions in most experiments.

As measured by extension during a 24-hour interval, the optimum concentration of 3-indolylacetic acid (IAA) was about 10 μg./ml. Higher concentrations were toxic (Fig. 1 and Table IV). With concentrations below 10 μg./ml. the response was linearly related to the logarithm of the IAA concentration (Fig. 1), as found by Galston and Baker (1951). The size of the response varied from experiment to experiment, but the optimum concentration of IAA was reasonably constant. The variation in magnitude of the response to IAA was largely due to the difficulty of regularly obtaining internodes of sufficient length and of the required degree of immaturity. In Fig. 1 the response to IAA in two experiments is shown; one with internodes still capable of vigorous growth, the other with somewhat more mature internodes. In most experiments it was possible to use material similar to that giving the greatest response in Fig. 1. Optimal concentrations of 2:4-

dichlorophenoxyacetic acid (2:4-D) and 1-naphthylacetic acid (NAA) were about 0·41 and 3·7 μg./ml. respectively (Fig. 1); in most experiments optimal 2:4-D produced as great an extension as optimal IAA, but NAA consistently produced less.

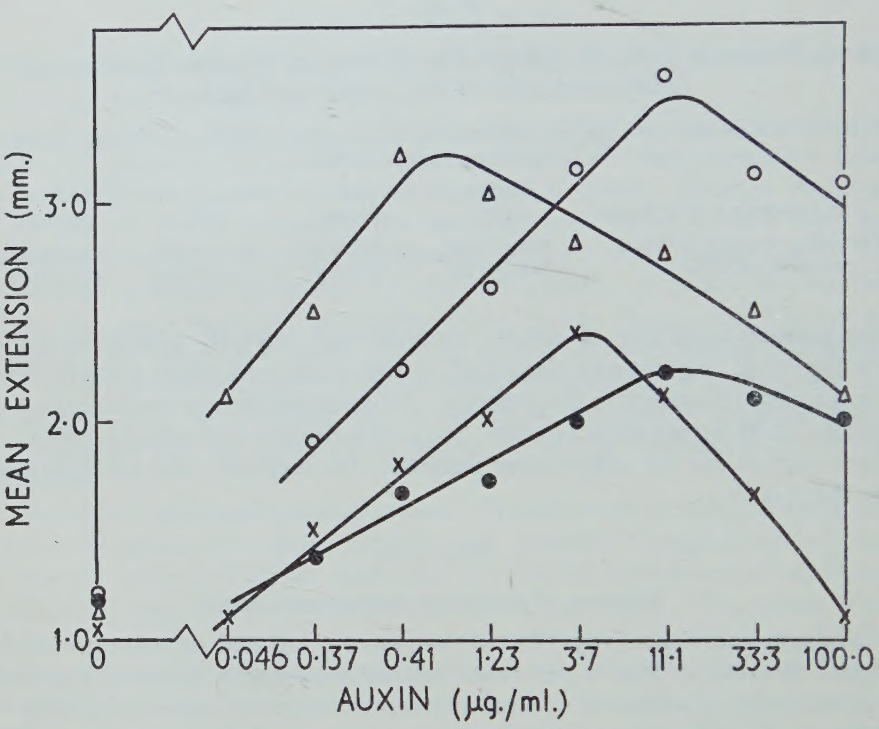

FIG. 1. Response of Meteor pea sections to IAA (—○—young internodes, —●— more mature internodes), NAA (—x—) and 2:4-D [—△—]. (Young internodes used except where specified to the contrary, all incubated in buffer + 2% sucrose.)

The time relations of the response to IAA are shown in Figs. 3 and 4. The growth-rate during the first 6 hours increased with increasing IAA concentration within the concentration range studied. The extension rate of all sections declined throughout the observation period. This fall was more rapid with the higher concentrations of IAA. Thus, with 100 μg./ml. IAA, which supported the highest initial rate of extension, the rate of extension fell so rapidly that at the end of the experiment the total extension was much less than that induced by 1 μg./ml. IAA. The fall in growth-rate in IAA was not due to exhaustion of the IAA supply; a trace of IAA could be found at the end of the experiment in solutions of 1 μg./ml. initial concentration, and 6 μg./ml. remained in the solutions of 10 μg./ml. initial concentration. These results are in general agreement with those of Bennet-Clark and Kefford (1954) for growth of oat coleoptile sections in comparable IAA concentrations.

Response of sections to gibberellic acid

Gibberellic acid had little effect on the extension of sections in buffer or in sucrose-buffer. In 12 out of 19 experiments in which GA was tested in buffer, at concentrations between 0·1 and 10 μg./ml., small increases in extension were observed; the increment due to GA never exceeded 0·3 mm. and statistical significance ($P < 0.05$) was only reached in two experiments. Similarly, in 6 experiments in which GA was tested in 2 per cent. sucrose-buffer, though small increments due to GA were always observed, they only reached significance ($P < 0.05$) once (Table IV).

In the presence of IAA, GA consistently and significantly increased the extension of sections. Tables II and III give the results and analysis of a factorial experiment in which sections were incubated in all possible combinations of IAA (10 μg./ml.), sucrose (2 per cent.) and GA (10 μg./ml.). Gibberellic acid produced significant effects only in the presence of IAA. There was a marked interaction between IAA and sucrose but, in this experiment, none between GA and sucrose; in other experiments significant interactions between GA and sucrose have sometimes been observed, and also significant second order interactions (GA × IAA × sucrose). Notwithstanding these differences between experiments, GA on all occasions caused highly significant extra extension in the presence of optimal IAA and sucrose. Similar results can be seen in Table V and others have been published elsewhere (Brian and Hemming, 1957*a*).

TABLE II

Mean Extension (mm.) of Meteor Pea Sections after 24 hr. incubation in Combinations of IAA (10 μg./ml.), Sucrose (2 per cent.) and GA (10 μg./ml.)

	No GA	+GA	Difference
Buffer only	1·71	1·37	−0·34
IAA	3·05	3·86	+0·81
Sucrose	1·75	1·78	+0·03
IAA and sucrose	3·91	4·48	+0·57

Significant differences: 0·47 ($P = 0.05$), 0·63 ($P = 0.01$), 0·81 ($P = 0.001$)

TABLE III

Main Effects and Interactions (mm. per section) Calculated from Data in Table II

Main effects		Interactions	
IAA	+2·17	IAA × sucrose	+0·26
Sucrose	+0·48	IAA × GA	+0·42
GA	+0·27	GA × sucrose	+0·03
		IAA × GA × sucrose	−0·15

Significant effects: 0·24 ($P = 0.05$), 0·31 ($P = 0.01$), 0·40 ($P = 0.001$)

Fig. 2 summarizes the results of three experiments in which GA was tested at several concentrations in the presence of 10 μg./ml. IAA and 2 per cent. sucrose. The size of the response varied from experiment to experiment but the same general relationships were observed in all. A near-maximal response was on all occasions obtained with 0·1 μg./ml. GA, but increase in concentration to 100 μg./ml. did not decrease response. A significant response from 0·01 μg./ml. GA was obtained on two occasions.

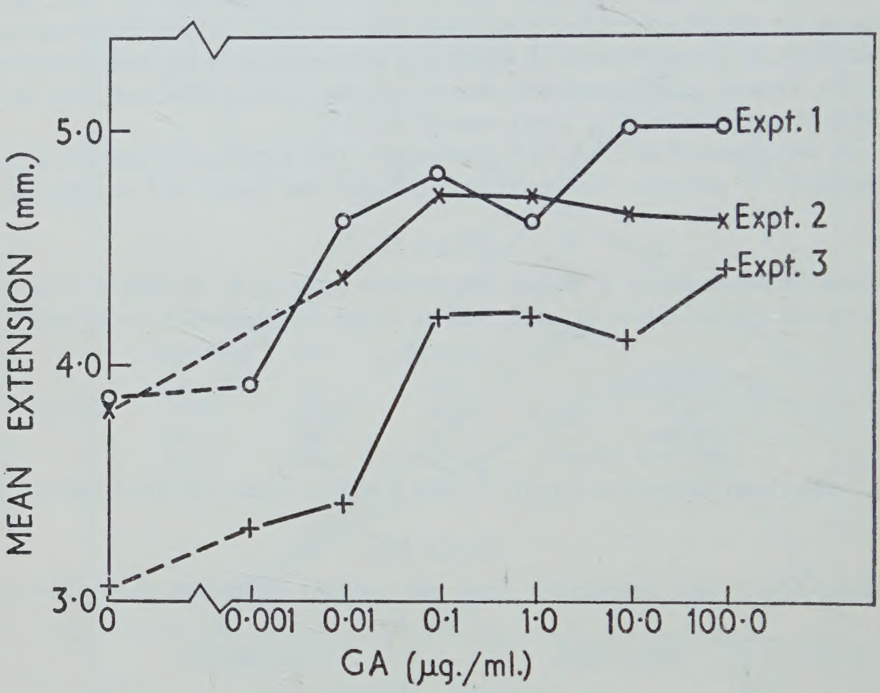

FIG. 2. Response of Meteor pea sections to GA in presence of 2% sucrose and 10 μg./ml. IAA. (Significant differences (P + 0·05): Expt. 1, 0·52: Expt. 2, 0·38; Expt. 3, 0·43.)

Table IV shows the response to 10 μg./ml. GA with IAA at several levels. Though on this occasion GA induced a significant extension even in the absence of IAA, much greater responses were produced in the presence of IAA, the biggest response being in the presence of 10 μg./ml. IAA, which we have previously shown to be near the optimum for this tissue.

Increased extension observed after an arbitrary time interval could be due either to an increased rate of extension during the period in which IAA exerts its main effect or to a prolongation of the phase of rapid extension. The results presented in Figs. 3 and 4 show that GA produces its greatest effect with IAA doses supporting most extension, viz. at 1 and 10 μg./ml. IAA rather than at 0 and 100 μg./ml. With the more favourable IAA levels it produced an effect over the whole or most of the growth period; in other words its effect was to increase the effect of IAA rather than to prolong it and GA did not prevent the normal decline in growth rate.

Thus GA and IAA are complementary in their effects. It seems reasonable to suppose that GA alone induces no extension of internode sections and that

Table IV

Mean Extension (mm.) of Meteor Pea Sections after 24 hr. Incubation in Sucrose-buffer and Various Concentrations of Gibberellic Acid (GA) and Indolylacetic Acid (IAA)

Concentration of GA (µg./ml.)	Concentration of IAA (µg./ml.)				
	0	1	10	100	1,000
0	2·11	2·59	4·19	2·91	0·29
10	2·33	3·22	5·55	4·44	0·32

Significant difference = 0·20 (*P* = 0·05), 0·26 (*P* = 0·01), 0·34 (*P* = 0·001)

Analysis of variance

Effects	Degrees of freedom	Sums of squares	Mean square
GA main effect . .	1	59·94	59·94*
IAA main effect . .	4	900·80	225·20*
Interaction . . .	4	35·68	8·92*
Residual error . .	290	43·51	0·150
Total . . .	299	1,039·93	—

* Variance ratio significant at 0·1% level.

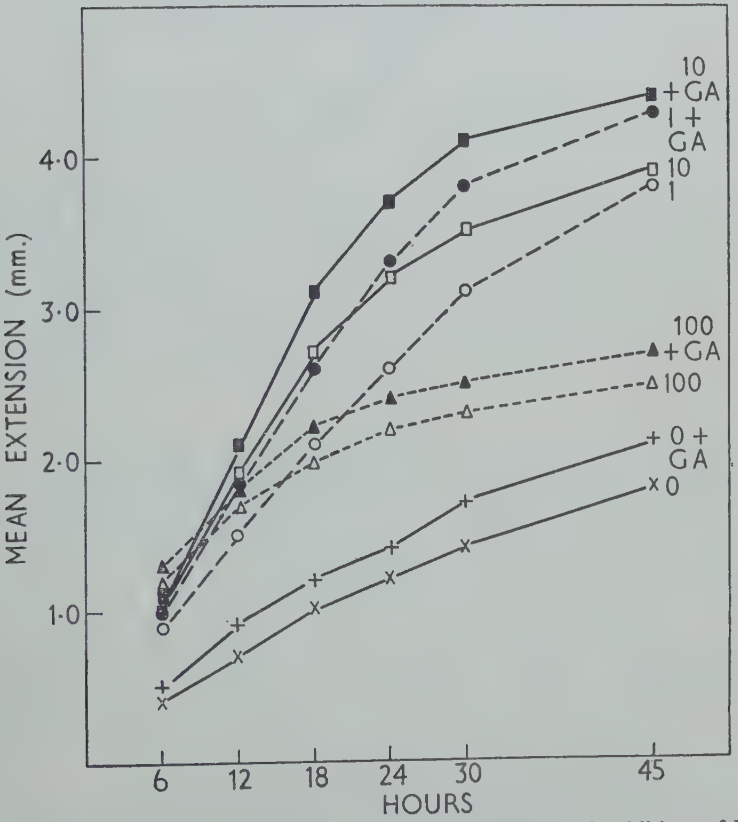

Fig. 3. Extension of Meteor pea sections in 2% sucrose-buffer, with additions of IAA and GA. (IAA concentrations are given in µg./ml. at right of figure; GA added at 10 µg./ml. where indicated.)

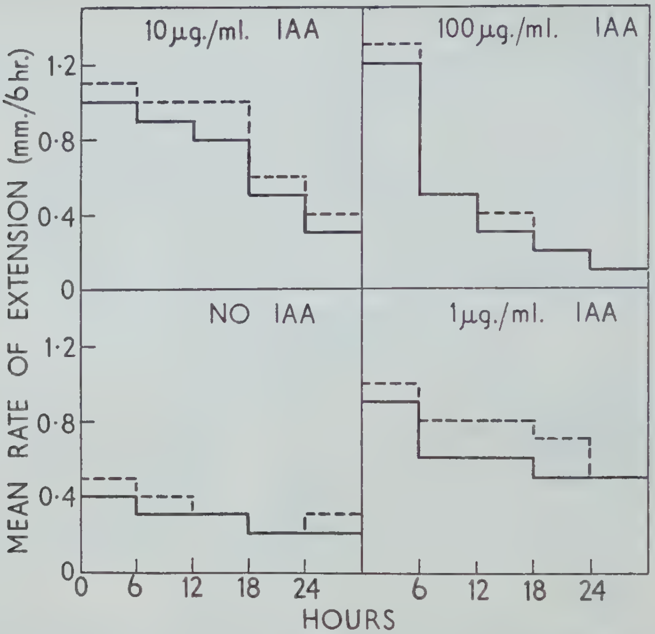

FIG. 4. Rate of extension of Meteor pea sections, derived from data of Fig. 3. (Full lines, IAA only; broken lines, with 10 µg./ml. GA.)

on those occasions when it does cause extension without exogenous IAA the effect is due to residual auxin in the section. Other auxins will replace IAA: significant increases in extension due to GA have been obtained in combination with 2:4-D (0·04–4·0 µg./ml.), 2-methyl-4-chlorophenoxyacetic acid (MCP) (0·04–4·0 µg./ml.) and NAA (0·4–4·0 µg./ml.). Indolylacetonitrile does not itself induce extension of internode sections, neither does it elicit a response to GA.

Relation of in vitro *observations to growth of intact plants*

The *in vitro* technique used was chosen in the hope that it would more closely reflect *in vivo* conditions than techniques based on the use of etiolated tissues in darkness. Nevertheless it is clear that internode sections behave differently, in some respects, from the same tissue in intact plants. For instance, Galston and Baker (1951) pointed out that sections respond to IAA but intact plants do not, or do so only to a very limited extent. This is not entirely surprising, since if auxin is produced mainly in the apical bud and leaves, the loss of these sources of auxin and the activation of oxidizing enzymes at the cut surfaces of the sections (Briggs *et al.*, 1955) would result in serious auxin-depletion in the section. More relevant to the present investigation is the observation that the response of sections to GA, even under the most favourable conditions, is far smaller than that of intact plants; extension of sections due to GA has rarely exceeded that of sections in IAA and sucrose by more than 40 per cent., whereas growth rates of comparable internodes of GA-treated intact plants may be two or three times that of untreated plants (Brian and Hemming, 1955). We have therefore sought to compare the behaviour of sections and intact plants somewhat more closely.

A batch of Meteor pea seedlings was raised in a greenhouse. Half were treated with GA (1 μg. in 0·002 ml. ethanol, applied to second true leaf) and half were untreated. At this time the mean length of internode 5, the uppermost visible internode, was 10 mm. From both sets of plants 20 were set aside and internode 5 was measured daily for 5 days. A further 20 plants of each set were removed daily to the controlled environment (C.E.) room (15° C., R.H. 80 per cent., continuous light intensity 800 f.c.) where section growth tests were carried out and the 24-hour growth increment of internode 5 under C.E. room conditions was obtained. At the same time, using the remainder of the plants as sources of material, fifth internodes were excised daily during the 5-day period of the experiment, and their extension measured during 24 hours in continuous light when floated on buffer solutions containing all possible combinations of 10 μg./ml. IAA, 10 μg./ml. GA, and 2 per cent. sucrose. When, in the later stages of the experiment, some internodes became too long to be conveniently handled, they were cut into two or more sections, and the increments of each of these sections were added. It was thus possible to measure the daily extension of (*a*) intact internodes in the greenhouse (15–20° C.) with normal daily lighting, (*b*) intact internodes from similar plants removed daily for 24 hours to C.E. room conditions, and (*c*) excised internodes in various solutions in C.E. room conditions. Of these, (*b*) and (*c*) were directly comparable. It might have been more satisfactory to raise the plant material entirely in the same C.E. room as used for the section tests, but sufficient room was not available.

Full results are given in Table V and the more important effects are also shown in Fig. 5. We should like to draw attention to the following points:

TABLE V

Mean Rate of Extension (mm./day) of Internode 5 of Meteor Pea Plants (a) Untreated and (b) Treated at Beginning of Experiment with 1 μg./plant GA

(Internodes in intact plants are compared with excised internodes incubated in various combinations of IAA (10 μg./ml.), GA (10 μg./ml.), and sucrose (2%))

	UNTREATED Period of observation (days)					TREATED Period of observation (days)				
	0–1	1–2	2–3	3–4	4–5	0–1	1–2	2–3	3–4	4–5
Intact plants										
In greenhouse . . .	3·2	3·5	3·6	2·4	1·8	8·0	13·0	17·0	15·1	7·1
In C.E. room . . .	3·5	2·0	2·3	2·2	1·7	7·8	10·0	13·6	11·0	3·8
Excised internodes										
Buffer only. . . .	3·3	2·8	3·1	2·6	2·7	3·9	3·8	4·1	3·6	3·2
Sucrose	4·3	4·0	4·3	2·6	2·5	4·1	4·6	4·6	3·4	3·0
IAA	6·1	7·7	8·4	8·5	8·0	7·0	12·1	14·4	16·1	11·3
IAA+sucrose . . .	6·6	8·8	10·0	9·5	8·5	8·0	13·6	16·2	17·4	12·7
GA	3·2	2·9	3·4	2·6	2·9	3·8	4·3	4·0	4·3	3·3
GA+sucrose . . .	4·0	3·2	3·8	2·8	2·9	4·8	4·1	4·2	3·1	3·1
GA+IAA	7·4	10·0	9·9	10·4	9·0	7·8	14·1	15·0	16·3	11·6
GA+IAA+sucrose . .	9·9	11·1	12·6	13·9	11·4	10·8	13·9	16·1	17·0	12·0

(*a*) The rate of extension of intact and excised internodes, from both untreated and GA-treated plants, tended to fall towards the end of the experiment; the fall was most noticeable in internodes from GA-treated plants and more noticeable in intact plants than in excised internodes. This was due to approaching maturity of the internode; in the case of GA-treated plants it was not associated with exhaustion of the GA applied to the plants, since the newly formed sixth internode continued to extend at a rate indicative of the presence of optimal GA concentrations. Internodes in intact plants grew more rapidly in the greenhouse than in the C.E. room.

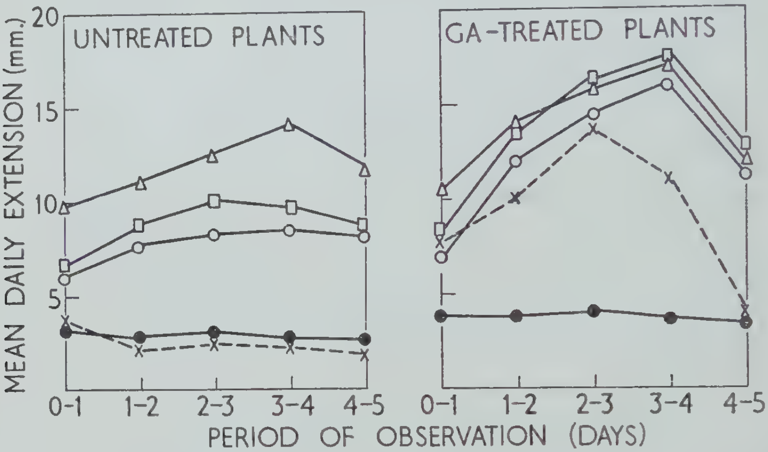

FIG. 5. Rate of extension of fifth internode of Meteor pea seedlings. (—x— in intact plants in C.E. room, —●— excised, in buffer; —○— excised, in buffer+IAA; —□— excised, in buffer+IAA+sucrose; —△— excised, in buffer+IAA+sucrose+GA.)

(*b*) Excised internodes from untreated plants behaved in the manner already reported for 5 mm. sections, viz. there was a large response to IAA, somewhat enhanced by 2 per cent. sucrose, and extension was increased by a further 30–45 per cent. by GA; as before GA only induced extension in the presence of IAA. In IAA-free solutions, excised internodes from GA-treated plants extended little more than those from untreated plants. If supplied with IAA, they extended more rapidly than those from untreated plants; at the time of most rapid extension, in the period 3–4 days from the beginning of the experiment, in IAA and sucrose they extended 83 per cent. more than comparable internodes from untreated plants. Addition of GA to the growth medium had no effect on extension of internodes excised from GA-treated plants except at the period 0–1 days when internodes were excised within an hour or two of treatment of the plants.

(*c*) Intact internodes of untreated plants extended at a rate slightly less than that of excised internodes in IAA-free solutions, i.e. far less than excised internodes in solutions containing IAA. Intact internodes of GA-treated plants, on the other hand, grew far more rapidly than excised internodes from similar plants in IAA-free solutions and approached the growth rate of excised internodes in IAA or IAA and sucrose.

This experiment has been repeated with essentially similar results. The implications of these observations are discussed at greater length in a later section of this paper, but one point requires mention at this stage, viz. that *in vivo* application of GA to plants produces a marked effect on extension of internodes subsequently excised from such plants only when those sections are incubated with IAA. Moreover, under such conditions, excised internodes extend as much as or more than intact internodes. This suggests that in intact plants, as we have shown to be the case in internode sections, GA depends upon the presence of IAA or other auxins for full manifestation of its effects on cell extension. We have further evidence supporting this view.

Though intact Meteor peas always respond to GA by increased stem extension, and occasionally respond to IAA by a small increase (Brian and Hemming, 1955), we have never found that application of combinations of IAA and GA elicit a more than additive response. In plants with the apical bud excised a positive interaction can be demonstrated. Tables VI and VII give the results of a factorial experiment in which intact and decapitated plants were compared, GA (0·1 per cent. in lanolin) or lanolin being applied to the base of the stem and IAA (0·1 per cent. in lanolin) or lanolin to the apical bud or cut stem apex. IAA alone had no significant effect on the extension of the upper internodes of intact plants but greatly stimulated extension of those of decapitated plants, whereas GA alone greatly increased extension of

TABLE VI

Mean Growth Increment (mm.) in 4 Days of Internodes 5 and 6 of Intact and Decapitated Meteor Pea Seedlings Treated with Combinations of IAA and GA in Lanoline

(Data are means of 20 plants; in decapitated plants the apical bud was removed by a cut at the node above internode 6)

Treatment	Intact	Decapitated
Untreated control (lanoline only)	9·2	7·9
IAA	11·8	13·0
GA	26·0	8·1
IAA+GA	23·0	17·2

Significant differences: 2·8 ($P = 0·05$), 3·8 ($P = 0·01$), 5·1 ($P = 0·001$).

TABLE VII

Main Effects and Interactions (mm./plant) Calculated from Data in Table VI

Main effects		Interactions	
IAA	+4·7	IAA × GA	−0·4
GA	+8·1	IAA × Decapitation	+3·7
Decapitation	−6·0	GA × Decapitation	−5·9
		IAA × GA × Decapitation	+2·4

Significant effects: 1·4 ($P = 0·05$), 1·9 ($P = 0·01$), 2·5 ($P = 0·001$).

intact plants but had no significant effect on decapitated plants. GA did increase extension of those decapitated plants treated apically with IAA. This result was in some respects unusual; in other experiments decapitated plants have responded significantly to GA alone, but the response was always smaller than that of intact plants and was always increased more than additively by apical IAA. Thus the response of an intact plant to GA depends to some extent on the presence of the apical bud and, since the apical bud can be partly replaced by IAA, its significance in the response of the plant to GA is probably partly attributable to its capacity to synthesize auxin.

MODE OF ACTION OF GIBBERELLIC ACID

This work was undertaken in order to clarify the relation between gibberellic acid and auxins in their effect on cell extension. We have shown that extension of pea internode sections in light depends primarily on auxin supply and that gibberellic acid only induces extension in the presence of auxin; under such circumstances the extension is greater than that which can be produced by auxin alone. Furthermore, there is evidence that this is also the case in the intact plant. In so far as GA is only active in the presence of an auxin it cannot itself be considered to be an auxin, as seemed probable from the many differences between gibberellic acid and the auxins in their spectrum of activity (Kato, 1953; Brian, Hemming and Radley, 1955).

If GA is not an auxin, how then does it enhance growth? There seem to be three main possibilities, two of which can with some confidence be rejected:—

1. *GA may combine with an auxin or auxins in the plant to form a more active compound.* This would explain the complementary action of auxins and GA. However it is unlikely that this is the case; we have shown that GA will enhance growth of internode sections in association with such diverse auxins as IAA, 2:4-D, MCP, and NAA, and chemical combination with all of these is unlikely; moreover, optimal extension is obtained with a mixture of 10 μg./ml. IAA and 0·1 μg./ml. GA, a molar ratio of about 50:1, which would seem to preclude chemical combination unless we assume that the molar ratios of IAA and GA are very different at the site of action.

2. *GA may accelerate growth by increasing the effective level of auxin in the plant, either by increasing the amount formed or by retarding its destruction.* This would seem at first sight to offer a reasonable explanation of the observation that intact internodes of untreated Meteor pea plants extend much less rapidly than excised internodes incubated in optimal IAA, whereas intact internodes of GA-treated plants grow at much the same rate as excised internodes in optimal IAA. However, one would also expect excised internodes from GA-treated plants to grow considerably faster in buffer alone than excised internodes from untreated plants, and this is not the case. In fact, excised internodes from GA-treated plants only grow appreciably faster than those from untreated plants if auxin is supplied in the incubation medium; thus GA must induce changes in the metabolism of the internode which make it more responsive to exogenous auxin. We have considered the

possibility that GA might reduce the rate of auxin destruction, but have been unable to detect any effect of GA on IAA destruction by pea internode sections or by IAA-oxidase preparations from pea-roots or etiolated pea-shoots. The complementary effect of GA with such auxins as 2:4-D and NAA, which are not so easily degraded enzymically as IAA, also reduces the likelihood that GA acts by inhibiting auxin destruction. Moreover, extensive investigations have yielded no evidence that auxin supply limits growth in dwarf peas (Abrams, 1953) and exogenously applied auxin does not appreciably accelerate growth of intact plants (Brian and Hemming, 1955).

3. *GA affects some metabolic process which normally limits growth even though auxin is present in non-limiting amounts.* The results presented in this paper give general support to this view and afford some indication of the kind of metabolic process involved.

The dose-response curve for GA in the *in vitro* internode section test (Fig. 2) and in other *in vitro* tests (Brian, Hemming, and Radley, 1955) is flat-topped, and the range between the lowest dose eliciting a response and the lowest dose producing a maximal response is very small. Such a dose-response relationship strongly suggests that some other factor is limiting the response. This limiting factor is neither auxin nor sucrose. On the other hand, a much greater range of response is obtained with intact plants (Brian and Hemming, 1955). Thus the limitation in response range is associated with detachment of the internode section from other parts of the plant. Similarly, increases of 300 per cent. in extension of a single internode of an intact plant may result from GA treatment, whereas the maximum increase in extension of an *excised* internode observed was 45 per cent.; here excision of the internode severely reduces the magnitude of the response to GA. Thus a comparison of conditions in intact and excised internodes may provide a clue to the nature of the physiological activity of GA.

We should not expect that effects of external conditions on the growth of sections would be the same as the effects on comparable tissues in intact plants. By the process of excision, such sections are removed from contact with meristematic tissues and with more mature tissues. As Brown, Reith, and Robinson (1952) have pointed out, growth of tissues used in excised form in *in vitro* techniques, in our case immature but non-meristematic internodes, is in the intact plant to some extent dependent on metabolites produced either in more mature tissues or in neighbouring meristematic tissues. The continually declining growth-rate of our internode sections after the first few hours, even in optimal IAA, GA, and sucrose, as compared with a steady rate of extension for several days in the intact plant (see Table V), is strongly indicative of the importance of such metabolic interchange. The greater effect of GA on the internode of an intact plant could be accounted for if it only secondarily affects growth of the internode, its primary effect being concerned with some process either in the apical bud or in the more mature tissues of the plant.

In our comparison of intact and excised internodes (Table V and Fig. 5) the most striking observation was that internodes in untreated intact plants grew far more slowly than was potentially possible (i.e. below the rate of extension of excised internodes incubated in IAA and sucrose), whereas internodes in GA-treated intact plants grew almost at the potentially possible rate. In another similar experiment the growth rate of intact GA-treated internodes actually exceeded slightly that of excised internodes in IAA and sucrose. For reasons already mentioned, it is unlikely that auxin or sucrose limit growth in the intact plant. We must therefore assume that there is, in the intact plant, an inhibitory system which limits growth-rate. Furthermore, since the excised internode is at least partially released from this inhibition by the process of excision, the complete inhibitory system cannot be an intrinsic part of the immature internode but must depend on translocation of some metabolite from other tissues. The effect of GA treatment can thus be envisaged as a neutralization of this inhibitory system. The greater response of intact plants to GA, as compared with that of excised sections, would then be expected, since the magnitude of the response to GA would depend on the activity or concentration of the inhibitory system.

We have so far referred to an 'inhibitory system' in general terms. It could be that we are dealing with a single growth-inhibiting substance, produced mainly outside the immature internode, whose production or effects are somehow prevented by GA. On the other hand various more complex systems might equally well be involved and there is no evidence at present available which enables us to define the 'inhibitory system' more precisely. However, the general concept is supported by genetical evidence (Brian, 1957), which also suggests that the growth response of dwarf pea plants to GA is due to neutralization of the effects of an inhibitor.

This view of GA activity is essentially that it plays no direct or positive part in cell extension, but that, by neutralizing an inhibition of extension, it releases the full potentialities of the auxins present. The dormancy-breaking effects of GA on potato-tubers (Brian, Hemming, and Radley, 1955; Rappaport, 1956) and some seeds (Lona, 1956; Kahn, Goss, and Smith, 1956) are compatible with such a view, all the more so since dormancy of this type has frequently been attributed to the presence of growth-inhibitory substances. GA also acts in a physiological sense as a reverser of inhibition in so far as it reverses red-light inhibition of stem internode extension in peas (Lockhart, 1956) and *Perilla ocymoides* (Lona and Bocchi, 1956).

There still remains one observation that needs explanation. It will be seen in Fig. 5 that the extension rate of excised internodes from GA-treated plants in IAA and sucrose reaches higher levels (maximum 17·5 mm./day) than excised internodes from untreated plants in IAA, sucrose, and GA (maximum 14·0 mm./day). Thus *in vivo* application of GA appears to be more effective than *in vitro* application. This is probably due to more even distribution of GA in the cells of the internode; the internodes from untreated plants were only exposed to GA during the 24-hour incubation period and such GA as does enter must do so by some form of diffusion, whereas the internodes of GA-treated plants had been exposed to GA for several days at the time the difference under consideration was most marked, the GA entering the tissues in this case by normal translocation processes. Also, at the beginning of any one period of *in vitro* growth, except the first, the internodes from GA-treated plants were longer than those from untreated plants and probably contained greater reserves of metabolites other than those (IAA, sucrose, and GA) supplies in the growth medium. Nevertheless, we cannot exclude the possibility that GA is more effective in the presence of unidentified metabolites more abundant in the intact internode than in the excised internode, but this does not invalidate the general scheme of GA activity which we have proposed.

GROWTH REGULATION IN THE PEA INTERNODE

In conclusion, we should like to put forward a few speculative proposals concerning growth regulation in the pea internode, which may also have wider significance. The differing response of plants to GA under different lighting conditions, and differential responses of wild-type and mutant-dwarf plants of various species, can best be explained by the assumption that hormones with physiological properties similar to those of GA occur naturally in plants (Brian, 1957*a*; Brian and Hemming, 1957*b*; Lockhart, 1956; Phinney, 1956). In this paper we have suggested that the physiological role of GA is most conveniently interpreted as neutralization of an inhibitor system, which otherwise restricts growth-rates to a level below that made potentially possible by the auxin available. Thus it seems possible that a three-factor system—auxin : GA-like hormone : inhibitor—regulates extension in the pea internode; such a system has also been proposed on genetical grounds (Brian, 1957). Direct biochemical evidence for the existence of 'GA-like hormones' has recently been found (West and Phinney, 1956; Radley, 1956; Lona, 1957), though none of these hormones has yet been isolated in greater quantities than spots on paper chromatograms. No direct evidence for the existence of an inhibitor system reversible by GA has yet been obtained. It is of course likely that more than one auxin is involved; similarly several inhibitors and several 'GA-like hormones' may be found, perhaps differing from plant to plant. But if some such three-factor system of growth regulators does in fact exist, and if it is found in other species as well as in the pea, then the differences in growth response to GA of different plant species, and the strikingly variable effects of GA on flowering in different species, could readily be explained by differences in activity of one or more of the components of the regulating system.

SUMMARY

1. Using an *in vitro* technique based on the extension in light of sections from growing internodes of green dwarf-pea seedlings, it has been shown that gibberellic acid (GA) only induces extension if an auxin is present. Most work has been done with 3-indolyl acetic acid (IAA) as the auxin; maximum

extension was obtained in a mixture of 10 μg./ml. IAA, 0·1 μg./ml. GA, and 2 per cent. sucrose. Higher levels of IAA were toxic but the GA concentration could be raised to 100 μg./ml. without reducing extension below the maximum. IAA could be replaced by 2:4-dichlorophenoxyacetic acid, 2-methyl-4-chlorophenoxyacetic acid, and 1-naphthylacetic acid. Extension of sections was not induced by 3-indolylacetonitrile (IAN), neither did GA have any effect in combination with IAN.

2. Extension of internodes in intact plants, using both untreated and GA-treated plants, was compared with the extension of internodes excised from such plants and incubated in solutions containing various combinations of IAA, sucrose, and GA. It was found that, although there was no evidence of limitation of growth by insufficiently high IAA or sucrose levels, intact internodes of untreated plants extended much less than excised internodes in IAA and sucrose. On the other hand, internodes from GA-treated plants grew as rapidly as excised sections from similar plants in IAA and sucrose.

3. On the basis of this and other evidence, it is suggested that GA neutralizes an inhibitory system in the pea shoot, which otherwise prevents the internode from extending to the full extent potentially allowed by the available auxin. It is also suggested that growth of the pea internode is normally regulated by a three-factor system, consisting of auxin, inhibitor, and a hormone with physiological properties similar to those of GA.

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The Saprophytic Status of *Fusarium oxysporum* Schl. Causing Vascular Wilt of Oil Palm

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ABSTRACT

The droplet plating method described, especially when used in conjunction with a baiting technique, facilitates the demonstration of *Fusarium oxysporum* in soil. This fungus was present in soil from wilt-free areas of oil palm plantations as well as in soil about wilt-diseased palms. Hyphal fusions between soil isolates and isolates from wilted palms could be demonstrated. The fungus persisted for periods of at least 1 year in naturally infested soils under a variety of moisture conditions; it also survived in inoculated alien soils for at least 1 year. Soil forms of the fungus had a high competitive saprophytic ability, competitively colonizing sterilized soils and sand, and organic materials added to soils and mixed cultures. The pathogenic isolate, as well as the soil isolates, exhibited characters belonging to soil-inhabiting fungi, namely continued persistance in soil: tolerance, in respect of growth and reproduction, to antagonism: and the ability competitively to colonize dead organic material in soils. The pathogenic form is considered to be a soil inhabitant.

INTRODUCTION

WARDLAW (1946, 1950) has described a vascular wilt disease of the oil palm (*Elaeis guineensis*) that is caused by a form of *Fusarium oxysporum* Schl. (Fraselle, 1951). The present paper deals with the saprophytic status in soil of this pathogen.

Knowledge of the persistence of root-infecting fungi in the soil lags behind knowledge of the parasitic behaviour of such fungi. Garrett (1956) has stressed this point and states that 'it is their saprophytic behaviour in soil that is the hidden phase in the life-cycle of most root-infecting fungi'. The distinction between the category of soil invading fungi and that of soil-inhabiting fungi is a result of their saprophytic behaviour. The first discussion of these two terms (Reinking and Manns, 1933) was based largely on members of the Elegans section of the genus *Fusarium* (= *F. oxysporum* sensu Snyder and Hansen). Some forms of *F. oxysporum* were said to be soil inhabitants; others, including the form later described as *F. oxysporum cubense*, were considered soil invaders. It should be noted that the differentiation into *forma* was based on morphology, a criterion now considered invalid for this purpose. In a taxonomic study of Fusaria in Canadian soils by Gordon (1956) *F. oxysporum* was the most prevalent species. Thus, on width of distribution the species may be regarded as soil inhabiting. It is possible, however, that forms (distinguished on physiological characters) may differ from each other in this

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respect. Anwar (1949) found that *F. oxysporum lini* acted as a soil inhabitant; Subramanian (1950) considers *F. oxysporum vasinfectum* to be soil inhabiting; Rishbeth (1955) favours the view that *F. oxysporum cubense* can act as a saprophyte in soil. Garrett (1956) in a general discussion on the saprophytic status of the vascular wilt fungi, concludes that their characters are those of soil-inhabiting fungi. On the other hand, Reinking and Manns's contention that the banana-wilt pathogen is a soil invader, is upheld by Stover and Waite (1954), who attribute to *F. oxysporum cubense* an expanding parasitic phase and a declining saprophytic phase. The fact that this fungus is known to survive for long periods in the absence of the disease could be explained by its presence as a parasite in disease-resistant varieties of the crop plant (Armstrong and Armstrong, 1948), or on wild host plants (Waite and Dunlap, 1953). An alternative explanation, which is due partly to Buxton (1954) and Rishbeth (1955), requires that nuclei conferring pathogenic properties become incorporated in a mycelium which contains other nuclei of a type allowing it to exist as a successful saprophyte, this established saprophyte acting as a carrier of the pathogen. Evidence exists supporting both these views.

MATERIALS

Samples of five soils were obtained from the Belgian Congo, and of two from Nigeria. 'Congo wilt soil' was taken from near the base of a palm with wilt symptoms on the Huilever estates at Yaligimba. 'Congo inoculum soil' was obtained from an area initially of healthy plantation but subsequently thoroughly inoculated with chippings from the trunk of a wilted palm. 'Congo soils *A*, *E*, and *Y*, were from plantations at Alberta, Elisabetha, and Yaligimba, respectively, from blocks in which wilt disease had not appeared. 'Nigerian wilt soil' was taken from near the base of a wilted palm on the Cowan Estate of Pamol Ltd. at Sapele; 'Nigerian soil *S*' was from the same plantations from a block where wilt had not appeared. All these soils were characteristic of the regions where oil palm is grown, namely, deep, acid sands of low base status, and with little organic matter (Bull, 1955). The soils were received in Britain about 2 weeks after collection.

Cultures of *F. oxysporum* freshly isolated from trunks of wilted palms were also received—six isolates (CW1 to CW6) from Yaligimba, and two (NW1 and NW2) from Sapele. Mass inocula from these initial isolates were transferred to tubes of autoclaved soil for maintenance with as little change as possible.

ISOLATION FROM SOILS AND CHARACTERIZATION OF CULTURES

Isolations were made in three series within 1 to 3 days of receipt of soil: firstly, particles of mineral soil were plated using Warcup's (1950) method; secondly, organic material from the soil (e.g. roots, chippings) was plated in the same way; and thirdly, pieces of organic material were mechanically macerated, and portions of the suspension plated (Stover and Waite, 1954). In each series three types of medium were used, Warcup's agar, Rose-bengal agar (Smith and Dawson, 1944), and 3 per cent. malt extract agar, at least 20 plates being poured in each series. Incubation was at 25° C. Plating by dilution was not attempted since Radford (1953) using this method on Nigerian soil from beneath a diseased oil palm failed to obtain isolates of *F. oxysporum*; similarly, Stover and Waite (1954) obtained but few colonies of *F. oxysporum* from rhizosphere soil of banana plants in an advanced stage of wilt disease, some attempted isolations giving completely negative results. Rishbeth, on the other hand, using the Warcup technique, appears to have had little difficulty in obtaining isolates of the fungus from soil bearing diseased banana plants.

The results of the platings are given here.

Congo wilt soil, Congo inoculum soil, and Nigerian wilt soil—*F. oxysporum* was detected in each soil by all three methods, but was most common on plates made by the Warcup method, especially when organic pieces had previously been surface-sterilized with calcium hypochlorite. Observations, and subsequent isolation of *F. oxysporum* was, however, difficult because of the vigorous growth of other fungi, particularly *Rhizopus nigricans*, *Thielaviopsis* sp., and *Trichoderma viride*. These fungi spread rapidly, formed a dense mat over the agar surface, and obscured the smaller colonies. In fact, pure cultures of *Fusarium* were obtained only with considerable difficulty.

[1]*Congo soils* A, E, *and* Y—*F. oxysporum* was found but infrequently in platings of soil *Y*, and not at all in those from soils *A* and *E*.

[1]*Nigerian soil* S—*F. oxysporum* was not seen in any of the platings.

The soils were examined for the presence of recognizable *Fusarium* conidia using the smear-slide method (Park, 1956). No recognizable conidia could be found, except in the Congo inoculum soil, in which occasional structures resembling microconidia of *F. oxysporum* occurred. In all soils thick-walled resting bodies, possibly of fungal origin, became stained.

In subsequent work the following isolates of *F. oxysporum* were used:

CW4 —from trunk of infected Congo palm,
CB11 —from mineral part of Congo wilt soil,
CC1 —from mineral part of Congo inoculum soil,
NW1 —from trunk of infected Nigerian palm,
NB4 —from mineral part of Nigerian wilt soil,
NA4 —from organic material in Nigerian wilt soil.

These six isolates were indistinguishable when compared together on potato plugs and on autoclaved rice.

The morphology of the three Congo isolates was compared by growing the organisms at 25° C. on plates of oat agar, potato-dextrose agar, Brown's agar, malt agar, Warcup's agar, and Czapek's agar. Colonies of identical appearance were obtained on oat agar and on Brown's agar; in the other cultures minor differences in the degree of radial striation, amount of aerial mycelium, and intensity of pigmentation were observed. The three Nigerian isolates exhibited greater uniformity when compared in this way.

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[1] Details of further tests on the presence of *F. oxysporum* in these soils are given later in this paper.

The growth compatibility of the isolates was examined by observing their ability to form mixed colonies, the Congo isolates and the Nigerian isolates being treated separately. Mixed spore suspensions were made in the following combinations:

CW4 +CB11
CW4 +CC1 —for Congo isolates
CB11 +CC1

NW1+NB4
NW1+NA4—for Nigerian isolates
NB4 +NA4

Plates of agar media as used in the foregoing morphological comparisons were separately inoculated with these six suspensions, and incubated at 25° C. The majority of the resulting colonies showed characters intermediate between those of the two component isolates. Some colonies approached the morphology of one component more closely than that of the other, but a significant feature here was that this dominance of characters was not prevailingly on the part of the soil isolates: in some combinations the wilt isolate was dominant. Thus, the characters of the pathogenic forms were not completely submerged in mixed colonies. Some plates, especially in subcultures of the mixed colonies, showed sectoring to one or both of the component isolates. Here again, the wilt isolates, as well as the soil isolates, reappeared, having retained their identity as far as could be determined from cultural features.

The ability to form mixed colonies is not necessarily indicative of close genetical relationship. The formation of hyphal anastomoses between two isolates is, however, said to indicate specific identity (Davidson, Dowding, and Buller, 1932), and this criterion has been used in taxonomic studies (e.g. Taschdjian and Muskatblit, 1955). Observations for hyphal fusions were made using the slide-culture method of Vernon (1931), each half of a tap-water agar droplet being inoculated with one of the isolates to be tested in each combination. The slides, supported in humid sterile Petri-dishes, were incubated at laboratory temperature. Permanent mounts, made from the cover slips and slides separately after growth, enabled observations at higher magnifications to be made. The following combinations were tested:

Congo isolates	Nigerian isolates	Congo against Nigerian isolates
CW4 +CB11	NW1+NB4	CW4+NW1
CW4 +CC1	NW1+NA4	CW4+NB4
CB11 +CC1	NB4 +NA4	CW4+NA4

In all combinations hyphal fusions were seen after 3 to 5 days.

METHOD OF DEMONSTRATION BY DROPLET CULTURES

On the isolation plates described earlier, colonies of *F. oxysporum* had appeared only in low to moderate frequency, and recognition of the presence of these colonies had been made difficult by the overgrowth of other fungi. Furthermore, specific identification of isolates on those plates was dependent on isolation in pure culture, and this was frequently unsuccessful in the case of suspected *Fusarium* colonies. For these reasons, none of these isolation methods is ideal for a simple and ready determination of the presence or absence of *F. oxysporum* in soil and organic materials. Plating from dilution suffered from the same disadvantages. Attempts to devise a culture medium selective for *F. oxysporum* were also unsuccessful. The suitability of the droplet-culture method of Curry (1949), designed for the isolation of slow-growing dermatophytes, was tested. In this method the droplet plates are prepared by pipetting 25–30 droplets of 3 per cent. malt agar on to the inner surface of a sterile Petri-dish lid, and inverting the lid over a base containing a few ml. sterile water to prevent drying of the agar. Droplet plates inoculated with particles of soil or plant residue (Congo wilt and inoculum soils and Nigerian wilt soil were used), and incubated at 25° C. gave *F. oxysporum* in over 90 per cent. of the inoculated droplets. The fungus appeared in 2–3 days and was still recognizable after 7 days. A range of other media was tested but none had any advantage over 3 per cent. malt extract agar (2 per cent. agar). There are several reasons why the technique is successful. Although other fungi in the inoculum are not prevented from developing, they produce, under these conditions, only a thin mycelium and do not markedly obscure the agar surface; moreover, the more vigorous fungi do not spread throughout the dish very easily. *F. oxysporum* produces compact groups of both microconidia and macroconidia on the agar, and often on the glass on which it frequently grows in advance of other fungi. These spore groups are readily located using low magnifications, and, the lid of the dish being inverted on the microscope stage, the high power objective can be swung round for a more detailed examination. In this mount the illumination can be made sufficiently critical to enable the delicate nature of the cross walls in the typical Elegans macroconidium to be seen and distinguished, e.g., from that of *F. solani*. The fungus can readily be obtained in pure culture by taking a loop of spores from a selected droplet, and streaking over the surface of a poured agar plate. In addition to providing conditions which favour sporulation of *F. oxysporum*, thus allowing easy identification, the droplet culture method is rapidly performed, permits ample replication, and requires little apparatus and incubation space. An added advantage is that the small area of the plates occupied by agar greatly reduces the probability of accidental aerial contamination of the medium.

SURVIVAL OF *F. OXYSPORUM* IN INFESTED SOILS

These experiments were designed to show whether *F. oxysporum* could survive for any length of time in soil, or whether it was present in the declining phase of a soil invader. The naturally infested soils used were Congo wilt soil and Nigerian wilt soil; Congo inoculum soil was also used. Tests for the presence of *F. oxysporum* in the mineral portion of the soils were made at regular intervals by the droplet culture method.

Moist soils

Survival under conditions favourable to microbial activity was examined by potting the soil in replicate sterilized 3 in. clay pots, and incubating in an electrically heated germinator cabinet (temperature 20°–31° C.; R.H. 80–95 per cent.). One series of triplicate pots of each soil was amended at three-monthly intervals with organic material in the form of autoclaved chopped grass leaf added to the surface as a 2 mm. layer, and then dug in to a depth of approximately 2 cm. The other series remained unamended. *F. oxysporum* continued to appear in more than 90 per cent. of inoculated droplets from all three soils up to 54 weeks. No differences between the amended and unamended series were apparent.

Dry soils

Under natural conditions the soils in oil-palm regions receive a supply of moisture throughout a large part of the year, but it was of interest to observe the effect of desiccation on *F. oxysporum* survival. Soils as received were air dried in sterile Petri-dishes and then poured into sterile, 2-cm.-diam. specimen tubes which were loosely plugged with cotton-wool. These were stocked in an incubating room (temperature 23–25° C.) receiving 18 hours artificial light at 150 foot-candles daily. Good survival of *F. oxysporum* continued in all three soils to 52 weeks.

Soils under water

The soils in oil-palm-growing areas do not normally become water-logged for long periods, and it would probably not be feasible to flood artificially. Nevertheless, since Stover (1954) has shown that *F. oxysporum cubense* may eventually disappear from soil under $\frac{1}{2}$ in. water in tumblers in the laboratory. it was of interest to consider this factor in the present case. The soils were packed loosely to a depth of 5 cm. in 2 cm.-diam. glass specimen tubes, and distilled water added to make a 1·5 cm. head of water above the soil surface. Loss by evaporation was maintained by additions of distilled water, so that the head varied between 1·0 and 1·5 cm. throughout the experiment. The tubes were kept in the incubating room. From the Congo soils *F. oxysporum* continued to appear in droplet platings up to 52 weeks. In the Nigerian wilt soil *F. oxysporum* was present in about 15 per cent. of the droplets at 20 weeks but could not be isolated nor seen on droplet plates at 24 weeks and subsequently. In view of some of the results which had been obtained using the baiting technique described later in this paper it was thought advisable to test the water-logged Nigerian soil by this method. At 31 weeks, about 1 g. of the soil was spread in the base of a sterile small Petri-dish, and propylene oxide-sterilized pieces of clover stolon added and left for 3 days. When these pieces were lifted and plated *F. oxysporum* appeared in at least 20 per cent. of the droplets. *F. oxysporum* could be isolated by this method up to 52 weeks. There was a profuse growth of green and blue-green algae in the tubes of this Nigerian soil, whereas in the Congo soil tubes algal growth was light.

Stover (1954) has observed that inocula of *F. oxysporum cubense* survived longer under water than in soil submerged in water. In connexion with the last experiment the ability of three of the Congo *F. oxysporum* isolates to survive in water was examined. Spore suspensions of isolates CW4, CB11, and CC1 were prepared. Sterile test-tubes (2 cm. diam.) were inoculated separately with 0·4 ml. of the appropriate spore suspension, and then made up to a depth of 10 cm. with sterile distilled water, and plugged with cotton-wool. Tubes were stored alongside those of the submerged soils, and the water level maintained at between 9 and 10 cm. Viable inocula have continued to be obtained from all tubes to 52 weeks. Morphological observations showed that microconidia and macroconidia were present in large numbers, and, although quite highly vacuolate, were viable.

F. OXYSPORUM AS A COMPETITIVE SAPROPHYTE

Two criteria that have been used for designating fungi as soil inhabiting are abundance or frequency in soil (often implying ease of isolation), and survival in soil. Neither criterion is completely adequate; a soil inhabitant must be an ecological saprophyte in being able to continue existence and activities in the soil, colonizing and utilizing decomposable dead substrata, and maintaining itself alongside other members of the soil population, without the necessity of an interposed parasitic phase. The properties that enable a fungus to behave in this way are included by Garrett (1950) in the term 'competitive saprophytic ability'. Garrett (1956 p. 130) uses the convenient shortened form 'saprophytic ability' but stresses that the concept is 'intended to express potential success in competitive colonization, rather than mere ability to utilize a particular substrate in pure culture'. As Garrett (1946) has pointed out, saprophytic ability in culture may not indicate saprophytic ability in soil. Unfortunately, competition is currently used in two senses and I should like to point out that in this context the word is used in the inclusive, Darwinian sense, which, in strict ecological terminology, is 'antagonism'. Odum (1954) and Clarke (1954) both review the possible interactions between pairs of species, and the general term 'antagonism' includes all associations in which at least one of the species is harmed. Antibiosis, competition, parasitism, and predation are subdivisions of antagonism, being the mechanisms through which the general phenomenon may be expressed. Competition in the specific sense is the *indirect* rivalry of two species for some feature of the environment that they both need, and that is present in limiting quantities. The concept of competitive saprophytic ability does not necessarily imply the operation of competition in this strict sense, but rather saprophytic ability under conditions of some sort of antagonism.

The competitive saprophytic ability of *F. oxysporum* was investigated by examining the ability of the fungus to colonize freshly cut potato tissue, dead plant tissues, and sterilized soil and soil-like substrata. The inocula used were the soils known to contain *F. oxysporum*, so that in these tests the species was subjected to antagonism from the other soil micro-organisms present in the Congo wilt, Congo inoculum, or Nigerian wilt soils used.

Potato tissue. Slices, 1 cm. × 1 cm. × 0·2 cm., were cut from the centre of stored but firm, potato tubers, and washed well in sterile water. Sample pieces showed no *Fusarium* on plating. Slices were buried in a vertical position to half their depth in moistened soil, which was incubated in the warm humid atmosphere of the germinator cabinet. Replicate slices were lifted after periods of from 3 to 7 days, when three stages of decomposition could be recognized: a lower brown and soft zone that was somewhat macerated (zone 1), a brown but firm zone (zone 2), and an upper cream-coloured firm zone (zone 3). The extent of each zone varied with the length of the incubation period. After surface-sterilization of the slices, several portions less than 0·5 mm. diam. were cut from each zone and plated in droplet culture. The slices from the two Congo soils showed *F. oxysporum* in all three zones after 3 days. The droplets inoculated with pieces from zones 2 and 3 were uni-fungal cultures of *F. oxysporum*, contaminated with bacteria, showing that the fungus had colonized in advance of other fungi in the soils. From the Nigerian wilt soil the slices showed *F. oxysporum* as the only fungus in zone 1 at 4 days; after 6 and 7 days other fungi such as *Trichoderma viride* had invaded zone 1, while *F. oxysporum* had advanced to zone 2, from which it was isolated in uni-fungal culture; zone 3 remained free from fungi during this time. Thus, *F. oxysporum* is highly successful at colonizing portions of potato tissue freshly cut from living tubers, but this is a rather specialized substratum, in that, even in moribund portions, there may still be some host resistance to overcome, and, although *F. oxysporum* may be an unspecialized parasite (Garrett, 1956 p. 65), it will have an advantage over obligate saprophytes that will be filtered out by the host resistance. Living storage tissue has successfully been used as a bait in soil for the unspecialized parasite *Thielaviopsis basicola* (Yarwood, 1946); in the present experiments the species of *Thielaviopsis* in Congo inoculum soil did not appear in the potato tissue until the stage marked by zone 3.

Dead plant tissues. The three materials used were partly decomposed oil-palm chippings obtained from Congo inoculum soil, portions of fresh stolon of *Trifolium repens*, and portions of the leaf of *Dactylis glomerata*. These plant parts were washed quickly in distilled water, and exposed to propylene oxide vapour for 30 hr., giving complete sterility. The test pieces were pressed on to the surface of a 5–10 mm. layer of the appropriate moistened soil in a Petri-dish. Dishes were incubated at 23°–24° C. Pieces were lifted, surface-sterilized, and plated 1, 2, 3, and 5 days afterwards. Within 2 days all the pieces had become colonized by *F. oxysporum*, and from the results (Table I) it is seen that the fungus is a vigorous colonizer, appearing early in the sequence along with such successful saprophytes as *Trichoderma viride* and *Rhizopus nigricans*. There was no specificity of *F. oxysporum* for oil-palm material, in fact, it was most successful on clover. This fact has been confirmed in repetitions and in other experiments. The fungus, then, shows a high degree of competitive saprophytic ability in the colonization of these organic substrata.

TABLE I

Colonization of Dead Plant Material from Soils

(*Fusarium* refers throughout to *F. oxysporum*)

Plant material	Days	Congo wilt soil	Congo inoculum soil	Nigerian wilt soil
Palm chippings	1	Rhizopus Trichoderma	Fusarium Thielaviopsis Trichoderma	No fungi
	2	Rhizopus Fusarium Trichoderma	Fusarium Thielaviopsis Trichoderma	Fusarium
Grass pieces	1	Rhizopus Penicillium Trichoderma	Rhizopus Fusarium	Fusarium Trichoderma
	2	Rhizopus Fusarium Penicillium Trichoderma	Rhizopus Fusarium	Fusarium Trichoderma
Clover pieces	1	Rhizopus Fusarium Penicillium Trichoderma	Rhizopus Fusarium Thielaviopsis	Fusarium

Colonization of sterilized soils. Soil dishes of the three soil types were sterilized by autoclaving for 30 minutes at a pressure of 15 lb. per sq. in. Three days after autoclaving each dish was inoculated near one side, within a small marked area, with a loop of the original (unsterilized) soil of the same type. The dishes were incubated at 23°–25° C., and observations made at weekly intervals by plating, in droplet culture, portions of soil taken at 1 cm. and 4 cm. from the edge of the inoculum area. After 4 weeks propylene oxide-sterilized pieces of clover stolon were placed over the soil at the 1 and 4 cm. marks, left for 3 days, then lifted and plated. The fungi appearing in these platings are listed in Table II. In addition to fungi, abundant bacteria, nematodes, and ciliates developed on the droplets. This experiment was of a twofold nature, testing firstly, colonization of the sterilized soil from the mixed natural soil population, and secondly, colonization of added organic material by organisms from this colonized soil. *F. oxysporum* was successful in both stages of the succession under conditions of antagonism which included intense bacterial development; it shared this property with only two other fungi, *Trichoderma viride* and *Thielaviopsis* sp. An objection to the acceptance of these results as an indicator of a general high competitive saprophytic ability for *F. oxysporum* might be that the soils were those known to contain the fungus previously, and might be regarded as specifically favourable to its growth and development.

TABLE II

Colonization of Autoclaved Soils from Soil Inocula, and Colonization of Clover Pieces from the Colonized Soils

(*Fusarium* refers to *F. oxysporum*)

	Congo wilt soil		Congo inoculum soil		Nigerian wilt soil	
Soil platings	1 cm.	4 cm.	1 cm.	4 cm.	1 cm.	4 cm.
1 week	Fusarium Trichoderma	Trichoderma	Cunninghamella Mucor Fusarium Thielaviopsis Trichoderma	Thielaviopsis Trichoderma	Trichoderma	No fungi
3 weeks	Fusarium Trichoderma	Fusarium Trichoderma	Cunninghamella Mucor Fusarium Thielaviopsis Trichoderma	Mucor Fusarium Thielaviopsis Trichoderma	Fusarium Trichoderma	Trichoderma
4 weeks	,,	,,	,,	,,	,,	Fusarium Trichoderma
Clover platings 4 weeks	Fusarium Trichoderma	Fusarium Trichoderma	Fusarium Thielaviopsis Trichoderma	Fusarium Thielaviopsis Trichoderma	Fusarium Trichoderma	Fusarium Trichoderma

Colonization of sand culture. A similar experiment was performed using artificial culture medium. 60-g. lots of washed quartz sand, sterilized in Petri-dishes by dry heat (160° C. for 6 hours), were moistened with 10 ml. sterile Erikson's (1947) artificial soil solution to which 0·01 g. per l. dextrose had been added. Inoculations and observations were made as in the previous experiment. The results of the platings are given in Table III, where, again, the presence of bacteria and animals is omitted. The sand substratum here is less specific, and more organisms did, in fact, appear in the platings than in those from the autoclaved soils, but *F. oxysporum* was again highly successful, and was the dominant organism in the clover platings. These experiments, then, indicate that *F. oxysporum* does possess a high degree of competitive saprophytic ability, both in respect of the colonization of decom-

TABLE III

Colonization of Sand Culture from Soil Inocula, and Colonization of Clover Pieces from the Colonized Sand

(*Fusarium* refers to *F. oxysporum*)

	Congo wilt soil		Congo inoculum soil		Nigerian wilt soil	
Sand platings	1 cm.	4 cm.	1 cm.	4 cm.	1 cm.	4 cm.
7 days	Rhizopus Fusarium Trichoderma	No fungi	Fusarium Trichoderma	Fusarium Trichoderma	Fusarium Trichoderma	Fusarium Trichoderma
17 days	Rhizopus Fusarium Trichoderma	Fusarium Trichoderma	Fusarium Thielaviopsis Trichoderma	Fusarium Trichoderma	Fusarium Trichoderma	Fusarium Trichoderma
Clover platings 4 weeks	Rhizopus Aspergillus Fusarium Trichoderma	Rhizopus Aspergillus Fusarium Trichoderma	Fusarium Thielaviopsis Trichoderma	Fusarium Trichoderma	Fusarium Trichoderma	Fusarium Trichoderma

posable substrata in soils, and in its growth through soils and soil-like matrices. Yet, although it has been shown that soil isolates have a genetical identity with pathogenic isolates, there is no certainty that the strains of *F. oxysporum* showing this success in saprophytic colonization are pathogenic to oil-palm plants.

An important feature of this series of experiments was that droplet platings of pieces of dead-plant materials that had been in contact with soil from one to several days gave even more easily recognizable colonies of *F. oxysporum* than did direct plating of the soil to droplet cultures. This was presumably because the inoculum potential of *F. oxysporum* was increased relative to that of other organisms present. Since this fact enables the droplet plating method to be made even more sensitive for recording the presence of *F. oxysporum*, the soils from plantation blocks not showing wilt disease, including those which had not given *F. oxysporum* by droplet plating, were again tested using this 'baiting' method. Propylene oxide-sterilized pieces of both grass and clover were used as baits, and were left in contact with the respective soils for 4 days before being lifted, and small portions plated in droplet culture. *F. oxysporum* was abundant in platings of both materials from Congo soils *A* and *Y*, and from Nigerian soil *S*. Colonies of the fungus appeared also in platings of these materials from Congo soil *E*, and, although here the fungus was not common, it could be identified in at least two droplets on each plate. Isolations were made, and one isolate from each soil selected for a test of hyphal fusions. Each isolate was tested against one Congo wilt isolate and one Nigerian wilt isolate as shown below.

CW4+Congo soil *A* isolate
CW4+Congo soil *E* isolate
CW4+Congo soil Y isolate
CW4+Nigerian soil *S* isolate

NW1+Congo soil *A* isolate
NW1+Congo soil *E* isolate
NW1+Congo soil *Y* isolate
NW1+Nigerian soil *S* isolate.

After 4 days hyphal fusions could be clearly observed between all pairs. Thus, *F. oxysporum* was present in areas of plantations where wilt had not occurred.[1] Specific identity of these soil isolates with the wilt isolates is shown by the hyphal fusions. The fungus was presumed to be present in these 'healthy' soils in low numbers, since it was not easy to demonstrate by direct plating of soil to droplets. The high numbers and relative ease of demonstration of the fungus in Congo wilt and inoculum soils and in Nigerian wilt soil may be related to the somewhat greater amounts of organic matter in these soils in the form of dead root tissue or as added chippings. Alternatively, wilt may be due to the initially higher numbers there.

COMPARISON OF WILT ISOLATE WITH SOIL ISOLATES

It is possible that soil isolates, and the strains showing high competitive saprophytic ability in soil, are not pathogenic. It is also possible that soil isolates of *F. oxysporum* are good competitive saprophytes, while wilt isolates, representing the pathogenic form, are not. Facilities for testing the pathogenicity for oil palm of the isolates were not available in this department. An attempt was made to estimate degrees of pathogenicity of the different isolates through tolerance to host resistance by measuring germination of conidia in extracts of oil-palm seeds, to test inhibition of the non-pathogenic forms. A similar procedure has been undertaken by Buxton (1954). No reliance, however, could be placed on the results from this method since the germlings, even after only 17½ hours at 25° C., produced further conidia, sometimes by budding, a phenomenon that characterizes the 'Junkultur' condition of species of *Fusarium* (Sherbakoff, 1915). This, of course, invalidates attempts to count the number of conidia added that have germinated. The process has also been observed by R. Caldwell working with strains of *F. oxysporum cubense* in this laboratory.

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[1] Further samples of soil have been obtained from wilt-free areas of the Calabar Oil Palm Estate of Pamol Ltd., an estate on which large areas have no history of wilt disease. Of 15 samples from 5 fields, 13 yielded isolates of *F. oxysporum*.

Tolerance in mixed culture. Another way of comparing wilt isolates with soil isolates was to examine them comparatively in respect of some of the characters of soil-inhabiting fungi. The author has stated elsewhere (Park, 1957) that in his view the limits of tolerance of a fungus for vegetative and reproductive activity in mixed cultures are important in determining whether the fungus can maintain itself in the culture, and has shown that this type of tolerance is wider for soil-inhabiting fungi than for exochthonous fungi. Tests of tolerance have probably more relevance to problems of the relationship between pathogenic forms and soil forms than do tests of the pathogenicity of soil isolates; negative results for the latter do not necessarily decide the problem, since, as discussed by Buxton (1954) and Rishbeth (1955), single soil isolates probably do not represent the full genetic expression of the wild population in respect of pathogenicity.

Tolerance tests of isolates CW4, CB11, and CC1 were made in mixed cultures on plates of Czapek's agar, 7–8 mm. deep. The antagonists chosen were *Mucor attenuatus* Linneman, *Aspergillus luchuensis* Inui, *Penicillium funiculosum* Thom, *P. jenseni* Zaleski, *Trichoderma viride* Pers., and *Rhizopus nigricans* Ehrenberg; all had been isolated by Warcup's method from Congo wilt soil. Agar plates, in separate series for each species of antagonist, were inoculated by flooding the surface with 1 ml. of a spore suspension of the antagonist, a seventh series was flooded with a mixed spore suspension containing spores of all six species. Excess liquid was decanted off, the plates dried at 30° C. for 3 hours, and then inoculated centrally with a loopful of a spore suspension of the test isolate of *F. oxysporum*. Plates were incubated at 25° C. for 8 days, after which portions of the culture from the inoculated area were plated, and portions from the 4 cm. region were examined microscopically for the presence of *Fusarium* macroconidia which would indicate that sporulation had taken place there. The results are shown in Table IV, from which it is clear that the pathogenic isolate CW 4 showed a degree of tolerance for growth and sporulation as high as that of the two soil isolates, while its tolerance for survival at the point of inoculation in the presence of *P. funiculosum* and *T. viride* was higher than that of soil isolate CB11. The success of each of the isolates in the presence of all six antagonists together, as compared with the lower degree of success in the presence of some of these individually may be explained by a lower antibiotic activity of the more virulent antagonists in the complete mixed culture.

TABLE IV

Tolerance of F. oxysporum *Isolates to the Presence of Antagonists on Agar*

	Fusarium Isolate Tested					
	CW4		CB11		CC1	
Antagonist	*Inoculum area*	4 cm.	*Inoculum area*	4 cm.	*Inoculum area*	4 cm.
Mucor attenuatus	+	sporulation	+	sporulation	+	sporulation
Rhizopus nigricans	+	sporulation	+	sporulation	+	sporulation
Aspergillus luchuensis	+	sporulation	+	sporulation	+	sporulation
Penicillium funiculosum	+	No Fusarium	—	No Fusarium	+	No Fusarium
Penicillium jenseni	+	sporulation	+	sporulation	+	sporulation
Trichoderma viride	+	No Fusarium	—	No Fusarium	+	No Fusarium
All 6 together	+	sporulation	+	sporulation	+	sporulation

+ = Fusarium isolated by plating
— = Fusarium not isolated by plating

A similar test was made in sand culture+artificial soil solution. The sterile cultures were inoculated all over with the spores of the appropriate antagonist, incubated at 23°–25° C. for 2 days, and then inoculated with a drop of a spore suspension of *F. oxysporum* in a marked area near one side of the dish. The plates were incubated at 23°–25° C., and observations made at weekly intervals by plating loopfuls of sand taken 4 cm. distant from the edge of the inoculated area. Table V shows the time taken for *F. oxysporum* to reach this point. After 4 weeks propylene oxide-sterilized pieces of clover were pressed into the sand over the inoculum, and also at the 4 cm. region, and left for 3 days, after which the bits were lifted and plated. Results of these platings are shown in Table VI. In this experiment isolate CW4, the

TABLE V

Time (in Weeks) Taken by F. oxysporum *Isolates to Reach a Distance 4 cm. from Inoculum, in Sand Cultures with Antagonists*

	Fusarium Isolate Tested		
Antagonist	CW4	CB11	CC1
M. attenuatus	1	4	4
R. nigricans	2	2	1
A. luchuensis	—	—	—
P. funiculosum	1	—	4
P. jenseni	4	2	3
T. viride	—	—	—
All 6 together	2	3	2

— = *Fusarium* did not reach 4 cm. distant from inoculum during 4 weeks wilt isolate, compares favourably with soil isolate CC1 in respect of its tolerance to antagonism, and shows a higher degree of activity under these conditions than soil isolate CB11. In all cases the pathogenic isolate had, at the least, survived in the inoculated area, and was there able to share in colonization of the decomposable materials.

TABLE VI

Colonization of Clover by F. oxysporum *from Sand Cultures with Antagonists*

Antagonist	Fusarium	Isolate	Tested			
	CW4		CB11		CC1	
	Inoculum area	4 cm.	Inoculum area	4 cm.	Inoculum area	4 cm.
M. attenuatus	+	+	+	+	+	+
R. nigricans	+	+	+	+	+	+
A. luchuensis	+	—	+	—	+	—
P. funiculosum	+	+	+	—	+	+
P. jenseni	+	+	+	+	+	+
T. viride	+	—	+	—	+	—
All 6 together	+	—	+	+	+	+

+ = *Fusarium* isolated by baiting
— = *Fusarium* not isolated by baiting

Survival in soil. Usually organisms alien to a particular soil do not survive for long on being added to that soil. The rate of disappearance from an inoculated alien soil was chosen as a criterion for a further comparison between the soil isolates of *F. oxysporum* and the pathogenic isolate. Two soils of an acid, sandy nature, low in organic content, and not containing any species of *Fusarium* in the natural flora, were selected for inoculation. These soils, one from Wigan, Lancashire, and one from Styal, Cheshire, were close in characters to the soil types found in African oil palm-growing areas. Two inoculation treatments were given. In one the soil was well mixed with 1 per cent. (by dry weight) of dried, chopped grass leaf that had been moistened, autoclaved, and inoculated with the appropriate isolate of *F. oxysporum* 2 weeks previously. The inoculated soil was potted up in 3-in. clay pots. In the second treatment the uninoculate soil was potted, and 4 ml. of a spore suspension of the *F. oxysporum* conidia pipetted on to the surface, and watered in. The pots were incubated in the germinator case. All three fungi have survived for 52 weeks in both soils and by both methods of inoculation. The isolates were readily demonstrated by direct droplet plating of mineral parts of the soils, even in the treatments inoculated with organic material. Sterile pieces of grass or clover placed on the surface of the inoculated soil rapidly became colonized by *F. oxysporum*, showing that the fungus exhibits its high competitive saprophytic ability even in this alien soil. Since all three isolates were equal in respect of the properties tested here, no contrast is possible, but the evidence shows that the wilt isolate exhibits the properties of a soil inhabitant in a degree at least as strong as those of the soil isolates.

DISCUSSION

The evidence presented shows that the species *F. oxysporum* exists in soil in oil palm-growing areas as a soil inhabitant. It can be isolated from a range of soils by a number of isolation methods, it survives in naturally infested soil under a variety of conditions, and it successfully colonizes dead materials added to the soil: in fact, all three criteria for soil inhabiting fungi are satisfied. An argument based only on this type of evidence, however, is subject to the criticism that it may apply only to certain soil-inhabiting forms, its extension to pathogenic forms of the species being unwarrantable. The distinctness of recognized *forma* in the natural habitat is the subject of divergent views. Snyder and Hansen (1940), while stating that 'all members of the section Elegans constitute one natural species group', admit the existence of forms which may differ in characters other than morphology. Miller (1946) subscribes to the contrasting opinion, that in the section Elegans there is one wild type which is the natural form of many different described species. Subramanian (1951, 1955) criticizes this hypothesis. Gregory (1952) contributes to a view that resembles Miller's in its implications, by suggesting that spores may, in Nature, germinate and fuse with an already existing mycelium, a process whereby the spores serve to disperse genes and transmit particular characters, rather than to increase the spatial range of the species. He compares the mycelium with a community rather than with an individual. This last hypothesis is currently acceptable. Jinks (1952*a*) has shown that heterokaryons do occur in the wild, and that they may have an advantage over homokaryons. Among the advantages are the increased heterokaryotic vigour (Dodge, 1942) and the presence of a pool of nuclei in the mycelium which allows the operation of a selective action by the substratum; heterokaryosis is thus adaptive in its significance (Pontecorvo, 1949; Jinks, 1952*b*; Brown and Wood, 1953; Buxton, 1954). These facts could complicate an analysis of the saprophytic survival of any particular form of the species *F. oxysporum*, since a form in itself possessing a low competitive saprophytic ability might persist as nuclei incorporated in a saprophytically successful mycelium. To assign a saprophytic status to the pathogenic form dealt with in the present work, however, is simpler, since it proved to have a tolerance to mixed culture conditions as high as that of the soil isolates tested. Moreover, it was as well able to survive in alien soils, and to colonize organic substrata in those soils, as were the soil isolates. The present pathogen, then, is considered a soil-inhabiting form.

Isaac (1953) working with species of *Verticillium* found that the most virulent pathogens were root-inhabiting forms, the others being soil inhabitants. The state described in the present paper is more in agreement with that described by Buxton and Richards (1955) who found no correlation between the degree of sensitivity to antagonism and the degree of pathogenicity in isolates of *F. oxysporum*.

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The Dependence of Net Assimilation Rate on Leaf-area Index

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With four figures in the text.

ABSTRACT

The leaf-area index (leaf area per unit area of land, *L*) of field crops of kale and sugar-beet was varied experimentally by removing different fractions of the plant population distributed uniformly through the crop. The net assimilation rate (*E*) was determined in subsequent periods of 10–14 days.

For kale, *E* decreased nearly linearly with increase of *L* throughout the range from 1 to 5. *E* of sugar-beet was less affected by change in *L* and was apparently not decreased until *L* rose above about 3.

Because of this dependence of *E* on *L*, the rate of dry-matter production per unit area of land, or crop growth-rate ($C = EL$), showed a curved relation to *L*; for kale it increased to a maximum when *L* was between 3 and 4 and fell again at higher values of *L*. Maximal *C* for sugar-beet occurred beyond the range of *L* tested, probably between $L = 6$ and $L = 9$. This optimal *L* for dry-matter production by sugar-beet crops probably lies near the upper limit of the current agricultural range, so there is little, if any, scope for increasing the dry-matter yield by further increase in *L*. For heavy kale crops *L* is already far in excess of the optimum, and it may be possible to increase the total dry-matter yield of kale by repeated thinning or defoliation to hold *L* near the optimum.

INTRODUCTION

THE total dry-matter production by a crop may vary through change in either the size of the photosynthetic system or in its activity, as well as in the length of the growth period during which photosynthesis continues. The photosynthetic capacity of crops expressed as leaf-area index (*L*; ratio of leaf area to land area) is much more variable in agricultural environments than the photosynthetic efficiency measured by net assimilation rate (*E*; rate of increase of dry matter per unit leaf area) (Watson, 1952); for example, variation of nutrient supply by fertilizer application, and differences between seasons in weather conditions, affect dry-matter yield mainly by varying *L*. Although small differences in *E* between varieties of some economic species have been demonstrated, there seems to be little hope of improving the efficiency of the photosynthetic process by selection or breeding, though it is possible that new species with higher *E* than those used at present could be introduced into agriculture. Within the limits set by natural climates to the length of the growth period, improvement of yield in existing cropping systems is likely to be brought about mainly by increasing *L*.

The extent to which the rate of dry-matter production can be increased by increase in *L* will depend on how *E* is affected by change in *L*. As *L* increases,

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mutual shading of the leaves would at some stage be expected to decrease photosynthesis by part of the foliage, and so to decrease E. This should occur even in bright sunshine because an increasing fraction of the total leaf area would be illuminated by light intensities below the saturation value. Also, crowding together more and more photosynthesizing leaves on unit area of land might decrease E by reducing the CO_2 concentration of the atmosphere in the crop.

To investigate the dependence of E on L it is necessary to vary L experimentally. There is no obvious way of suddenly increasing the leaf area of a field crop, but it can be decreased by cutting off leaves or by removing whole plants. Removal of individual leaves would be laborious, and complicated by the need to ensure that the same proportions of leaves of differing age and morphological status were taken. It would also be objectionable because it would alter the leaf-area ratio (total leaf area: total plant dry weight) and so change E by changing the balance between photosynthetic gain and respiratory loss of dry matter independently of change in the rate of photosynthesis. It was therefore decided to vary L by removing whole plants by differential thinning of the crop.

The crops were grown in close spacing with liberal amounts of fertilizer to produce as large a leaf-area index as possible, and at the beginning of each experimental period a variable fraction of the plant population distributed systematically through the crop was removed from different plots. This produced crops consisting of plants with uniform previous history and leaf-area ratio but differing in L. Of course, uniformity of the plant material did not persist through the following period in which the dry-weight increment was measured, because the amount of growth per plant varied with the plant population, but the period of 10–14 days was too short for large differences to develop.

There are other ways of producing variations of L by varying the date of sowing or by application of fertilizers, but such treatments might change E by direct effects of age or nutrition on the photosynthetic mechanism independently of any effect of varying L.

ARRANGEMENT OF EXPERIMENTS

Kale (variety: Garton's thousand-headed) was used for most of the experiments, but two were done on sugar-beet (variety: Kleinwanzleben E). These species were chosen because they have large entire leaves convenient for leaf-area estimations by the punching method (Watson and Watson, 1953). Both crops were sown in rows spaced 18 in. apart, and the plants were thinned as accurately as possible to 4 in. apart in the rows; the mean number of plants per acre was 88 thousand for kale and 85 thousand for sugar-beet. All the crops received 10 cwt. per acre of a granular compound fertilizer supplying 0·7 cwt. N, 0·7 cwt. P_2O_5, and 1·0 cwt. K_2O per acre in the seed-bed. The kale seed was treated with a B.H.C. seed-dressing and the seedlings were dusted with 5 per cent. D.D.T. dust to control flea beetles; fortunately, the crops were almost free from other insect pests and diseases. The sugar-beet were sown late, in May, to produce maximal leaf area (Watson and Baptiste, 1938), and with the same object a top dressing of 3 cwt. nitrate of soda (0·5 cwt. N) per acre was given in June. Nearly 10 per cent. of the plants were infected with beet yellows virus by early September. Weeds in both crops were controlled by repeated hoeing.

The dimensions of the plots were 5 rows × 6 ft. or 6 ft. 8 in. (i.e. 18 or 20 plants per row, approximately). The area per plot was therefore 4·18 or 4·65 m^2. and the number of plants per plot 90 or 100 approximately. One row on each side of every plot, and a strip at each end in the direction across the rows, were treated in the same way as the plot, but not harvested.

The earlier experiments comprised three treatments, viz. 0, $\frac{1}{3}$, or $\frac{2}{3}$ of the plants removed, arranged in 8 randomized blocks of 3 plots. Later experiments, when growth of the unthinned crop made it practicable to vary L over a wider range, included four treatments, viz. 0, $\frac{1}{4}$, $\frac{1}{2}$, or $\frac{3}{4}$ of the plants removed, arranged in 5 or 6 randomized blocks of 4 plots.

In Exp. 5 on kale, an attempt was made to extend the range of L still further by application of additional nitrogenous fertilizer (N). The treatments consisted of all combinations of (*a*) O *v*. N with (*b*) 0, $\frac{1}{4}$, $\frac{1}{2}$, or $\frac{3}{4}$ of the plants removed, arranged in 4 randomized blocks of 8 plots. The N plots received 3 cwt. 'Nitrochalk' (0·5 cwt. N) per acre on 25 June, but as this treatment had no detectable effects on either dry weight or leaf area, it is ignored in the following discussion and Exp. 5 is treated as if it consisted of 8 replicates of the 4 variations in plant population. The details of each experiment are given in Table I.

SAMPLING PROCEDURE

Plots were marked out at the beginning of an experiment, and the appropriate fraction of plants was removed from each plot by uprooting them. Occasionally a kale stem broke and the root was then dug with a hand fork. A few sugar-beet plants were so firmly rooted that they could not be pulled but had to be lifted with a fork. Where one-third of the plants were to be removed, the first was selected at random in the first three plants of an outside row, and every third plant was then taken, moving up and down the rows over the plot. A similar procedure was followed for the other thinning treatments. Where one in every three, or one in every four, of the plants were taken, the spacing of the remainder in the rows was not uniform, one wide spacing alternating with one or two narrow spacings, but removal of $\frac{1}{2}$, $\frac{2}{3}$, or $\frac{3}{4}$ of the plants left a uniform spacing 2, 3, or 4 times the original spacing respectively.

The plants removed from each plot at the time of thinning, sample I, were counted and the leaf laminae were cut off. The small lobes of lamina on each side of the petioles of the kale leaves were ignored. The detached laminae were weighed, thrown into a heap at random, and discs were cut from them with a circular punch thrust into the heap. A 2-in.-diameter punch was

TABLE I

Details of Experiments

	Date of sowing	Date of thinning	Exp.	Treatments*	Number of replicates	Area per plot, m.^{2}	Mean number of plants per plot before treatment	Dates of sampling	
								Initial	Final
1953 Kale	17 March	19 May	1	A	8	4·2	91	25–26 June	9–10 July
1954 Kale	23 March	7–10 May	2	A	8	4·2	94	18 June	28–29 June
			3	A	8	4·2	93	2 July	12–13 July
			4	B	5	4·6	107	16 July	26 July
			5	B	8†	4·6	100	29–30 July	10–11 August
			6	B	5	4·6	99	16 September	29–30 September
Sugar-beet	8 May	15–21 June	7	B	6	4·2	89	5 August	16–17 August
			8	B	6	4·2	83	25 August	6–7 September

* A = 0, $\frac{1}{3}$, or $\frac{2}{3}$ of plants removed.
B = 0, $\frac{1}{4}$, $\frac{1}{2}$, or $\frac{3}{4}$ of plants removed.

† See p. 39.

used for the kale leaves, but with the smaller sugar-beet leaves this gave too high a proportion of incomplete discs, so a smaller punch of $1\frac{1}{4}$-in.-diameter was used. The number of discs in a weighed sub-sample (200 g. for kale; 150 g. for sugar-beet) was counted, including all incomplete discs larger than a half but excluding smaller ones. The area per unit weight of lamina was calculated from the number of discs, the known area per disc, and the weight of the sub-sample; it was then multiplied by the initial fresh weight of lamina and divided by the number of plants to give the mean leaf area per plant of sample I. The sub-sample of discs was dried at 100° C. to determine dry matter per cent. of fresh weight. Soil adhering to the kale roots was removed by beating them on a hard surface, and the sample of stems with attached petioles and roots was weighed. Twelve of the stems drawn at random from the sample were cut up in a chaff-cutter and a 200-g. sub-sample was taken for dry-matter estimation.

The petioles and crown were cut off each sugar-beet plant and the storage roots were scrubbed clean. The petioles and roots were weighed and sub-sampled for determination of dry matter per cent. of fresh weight. From the number of plants in the sample, and the fresh weights and dry-matter percentage of the various plant parts, the mean dry weight per plant of sample I was calculated.

The plants remaining on each plot (sample II) were harvested at the end of the experimental period of 10–14 days, counted, and treated in the same way as sample I. The total leaf area and total dry weight of sample II were calculated from the count of leaf discs and the fresh weights and dry-matter contents of the several plant parts. Estimates of initial leaf area and dry weight of the plants forming sample II were obtained by multiplying the mean leaf area per plant and mean dry weight per plant of sample I, taken from the same plot, by the number of plants in sample II. On the unthinned plots all the plants were left for harvest at the end of the experiment, and there was no sample I. The initial leaf area and dry weight of sample II for each of these plots was determined from the average of the estimates of mean leaf area and dry weight per plant of sample I from all the other plots in the same block.

The primary data therefore consist of initial and final dry weights and leaf areas per plot of the plants harvested at the end of the experimental period. For uniformity, the dry weights have been expressed as yields in g. per m.^{2} of land surface, and the leaf areas in m.^{2} per m.^{2} of land surface, i.e. as leaf-area index.

CALCULATION OF GROWTH FUNCTIONS

The mean net assimilation rate during the period of an experiment was estimated for each plot as

$$\frac{(W_2-W_1)(\log_e L_2-\log_e L_1)}{(t_2-t_1)(L_2-L_1)},$$

where W_1 and W_2 are initial and final total dry-weight yields respectively of sample II, L_1 and L_2 the initial and final leaf-area indices, and t_2-t_1 the length of the interval in weeks. The mean rate of increase of dry-weight yield in g. per m.^{2} of land area per week is

$$\frac{W_2-W_1}{t_2-t_1}.$$

It is suggested that this important attribute of crop growth, hitherto without a name, be called the *crop growth-rate* (symbol C). The mean leaf-area index over the experimental period (L) was calculated as

$$\frac{L_2-L_1}{\log_e L_2-\log_e L_1}$$

for the sake of consistency, so that for individual plots $C = EL$, but the treatment means of L calculated in this way were almost identical with arithmetic means of L_1 and L_2. Mean L is the independent variate whose influence on E is to be investigated. Relative leaf-growth rate per cent. was calculated as

$$\frac{\log_e L_2-\log_e L_1}{t_2-t_1}\times 100.$$

The treatment means of the primary and derived data and their standard errors for all the experiments are given in Table II.

Growth curves of total dry weight and leaf-area index of the unthinned crops (Fig. 1) show that the estimated initial and final values for sample II in each experiment plotted against time fell close to the same curve, indicating that the estimates of initial weights and areas from the thinned plants introduced no serious bias in comparison with values obtained by harvesting whole plots. The points for the 1953 crop were very close to the growth curves for the 1954 crop. The dry-weight yield of kale was still increasing at the last harvest in September by which time it had reached more than 6 tons per acre, and many of the plants were 7 ft. tall. The dry-weight yields of sugar-beet were less than those of kale at comparable times, but the difference steadily decreased with time because of the more rapid growth-rate of the sugar-beet. For kale, L increased to a maximum of 5·5 in mid-August and subsequently decreased to 5 in September, and for sugar-beet, L increased from 4 in mid-August to more than 6 in early September. These high values of L satisfied the requirement of permitting experimental variation of L over a wide range.

SOURCES OF ERROR

Uprooting the plants on the thinned plots at the beginning of each experiment presumably disturbed the roots of the neighbouring plants left for later harvest, but there was no evidence that the growth of these was adversely affected. The plants on the thinned plots were never seen to wilt; the summer of 1954 was unusually wet and the plants were never subjected to severe water stress. In Exps. 4 and 5 some of the very tall plants on the severely thinned plots fell or leaned over through lack of support and protection from wind by their neighbours. Any harmful effect of thinning on growth in dry weight of the remaining plants would reduce E for thinned plots compared with unthinned plots, i.e. it would tend to minimize any decrease in E caused by increase in L.

Though care was taken to avoid damaging the crop during thinning, leaves were occasionally broken off. Fig. 1 suggests that the amount of dry matter lost from the thinned plants in this way was small because the estimates of the initial weights calculated from the thinned plots were not consistently below the growth curve of the final dry weight of unthinned plots. A small loss of leaves from the plants left after thinning would presumably have negligible effects on the estimates of E.

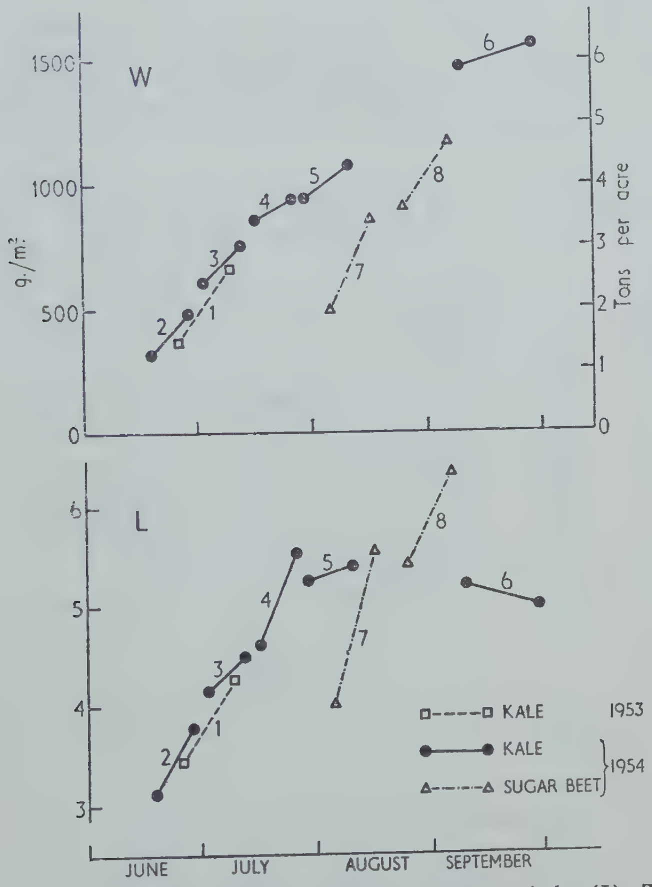

FIG. 1. Growth curves of yield of dry matter (W) and leaf-area index (L). The lines join points representing values for the unthinned crop calculated from the plants removed at sampling I and from the unthinned plots of sampling II in each experiment. The numbers refer to the experiments listed in Table I.

An unavoidable source of error in growth studies on field crops is that the whole root system cannot be recovered, and at times, when the dry weight of the roots is increasing, this causes E to be underestimated (Williams, 1946).

RESULTS

In Exps. 1–5 on kale, E was consistently decreased by each increase in L throughout the ranges tested (Table II). In all these experiments the differences between the means of E corresponding to the highest and lowest values of L were significant and so also were many of the differences for successive steps in the scale of increasing L. The standard errors of E in Exp. 6 were much higher than in the others, because this experiment was done late in the season when the plants were larger and growing more slowly, so that the dry-weight increments during the experiment were less accurately

TABLE II

Treatment Means of Leaf-area Index (L), Total Dry-matter Yields (W), Net Assimilation Rate (E), and Crop Growth-rate (C)

Exp.	Treatment (fraction of plants removed)	Remaining plants per m.^{2}	L			W g./m.^{2}		E g./m.^{2}/week	C g./m.^{2}/week
			Initial	Final	Mean	Initial	Final		
				Kale					
1953	2/3	7·7	1·2	2·0	1·6	121	303	59	91
1	1/3	14·0	2·2	3·1	2·6	243	497	48	126
	0	22·1	3·4	4·3	3·8	367	663	39	148
	S.E.	..	..	..	..	..	..	2·6	7·8
1954	2/3	8·3	1·2	1·6	1·4	122	215	45	63
2	1/3	14·9	2·1	2·8	2·4	208	354	42	99
	0	22·0	3·1	3·8	3·4	315	483	33	114
	S.E.	..	..	..	..	..	..	3·3	6·9
3	2/3	7·7	1·4	1·8	1·6	207	316	46	73
	1/3	14·1	2·8	3·0	2·9	390	524	32	89
	0	22·2	4·1	4·5	4·3	606	755	23	99
	S.E.	..	..	..	..	..	..	4·4	10·0
4	3/4	5·9	1·2	1·7	1·4	221	319	48	69
	1/2	11·8	2·4	3·1	2·7	436	606	44	119
	1/4	17·1	3·6	4·3	4·0	678	793	21	81
	0	22·4	4·6	5·5	5·0	856	941	12	60
	S.E.	..	..	..	..	..	..	5·9	14·5
5	3/4	5·9	1·4	1·8	1·6	253	403	54	88
	1/2	10·8	2·6	3·2	2·9	509	682	34	101
	1/4	15·6	4·0	4·4	4·2	699	923	32	130
	0	21·0	5·2	5·4	5·3	947	1,080	14	78
	S.E.	..	..	..	..	..	..	6·4	21·4
6	3/4	5·5	1·2	1·4	1·3	377	466	33	46
	1/2	10·4	2·6	2·6	2·6	701	791	18	47
	1/4	16·1	4·2	3·9	4·1	1,189	1,349	20	83
	0	20·9	5·2	5·0	5·1	1,478	1,565	8	44
	S.E.	..	..	..	..	..	..	13·7	28·6
				Sugar-beet					
7	3/4	5·7	1·1	1·4	1·2	144	262	57	71
	1/2	10·8	2·0	2·8	2·4	255	512	66	156
	1/4	15·6	2·9	4·0	3·4	349	644	54	179
	0	20·6	4·0	5·5	4·7	495	855	47	217
	S.E.	..	..	..	..	..	..	5·2	13·9
8	3/4	5·2	1·5	1·8	1·6	258	357	34	56
	1/2	10·1	2·8	3·2	3·0	458	650	36	107
	1/4	15·2	4·1	4·8	4·4	695	925	29	128
	0	19·4	5·4	6·3	5·8	910	1,175	25	148
	S.E.	..	..	..	..	..	..	7·4	24·1

determined. None of the differences between treatment means of E in Exp. 6 were significant, but the results are consistent with those of the other experiments in showing that E decreased with increase in L, though not so regularly over the whole range of L.

E for sugar-beet was much less affected by change in L than E for kale (Table II). In both sugar-beet experiments the mean values for E were higher when half the plants were removed ($L = 2{\cdot}4$ in Exp. 7 and 3·0 in Exp. 8) than when $\frac{3}{4}$ or $\frac{1}{4}$ of the plants were removed, and the unthinned plots gave a lower E than the others. However, the only significant effects in Exp. 7 were the decrease in E corresponding to increase in L from 2·4 to 4·7, and the difference between the means of E for the two higher and the two lower values in the L scale ($61{\cdot}5-50{\cdot}0 = 11{\cdot}5 \pm 5{\cdot}2$). There were no significant differences in Exp. 8.

When linear regressions were fitted to the treatment means of E for each experiment (Table III), all the regression coefficients were negative and those for Expts. 1–5 were significant. The coefficients for sugar-beet were much smaller than for kale, and were not significant in either Exps. 7 or 8.

The relation of E to L is unlikely to be strictly linear over the whole range of L. When L is less than unity, or does not much exceed it, there should be little mutual shading of leaves and E should therefore not be much affected by change of L within this limit. The results for sugar-beet might be interpreted as indicating that E was independent of variation in L over a still wider range from 0 to about 3, and began to decrease only at higher values of L. However, a quadratic term introduced into the regression failed to account for a significant fraction of the variance of E in any of the experiments. The results therefore show that E for kale decreased with increase of L at a rate that did not vary detectably over the range tested, i.e. at an apparently constant rate. There was evidence of a similar change in E for sugar-beet but it was much smaller than for kale, and the experiments were not sufficiently accurate to establish it definitely.

When the regression lines are plotted (Fig. 2*a*) they do not fall into an orderly system, partly because the absolute magnitudes of E varied with short-term and seasonal fluctuations in environmental factors. If the scales of E are adjusted to make the regressions coincide at a point in the range of L where mutual interference between leaves is likely to be small, the effect of varying climatic factors can be eliminated. This is done in Fig. 2*b* where the values of E on each regression line are plotted as per cent. of the value for $L = 1$. The relative slopes of the regression lines for kale tend to become steeper with age of the crop. The line for Exp. 2 in June 1954 lies above the rest and next below it is that for Exp. 1 in early July 1953. The other lines, for experiments done later in the season, all fall more steeply and almost coincide except the one for Exp. 4 which is below the rest. These differences in the slope of the regression lines were not reliably established, because the regression coefficients expressed as per cent. of the value of E for $L = 1$ (Table III) did not differ significantly. The regression lines for the two

TABLE III

Linear Regression Coefficients of E on Mean L

	Kale						Sugar-beet	
Exp.	1	2	3	4	5	6	7	8
Regression coefficient	−8·8±1·6*	−5·9±2·3†	−8·3±2·3‡	−10·8±2·2*	−9·7±2·3*	−5·5±4·8	−3·9±2·0	−2·2±2·3
Regression coefficient per cent. of E when mean $L = 1$	−13·9±2·6	−12·1±4·7	−16·8±4·6	−19·4±3·9	−16·8±4·0	−17·0±14·9	−6·1±3·2	−6·0±6·2

† $P < 0·05$. ‡ $P < 0·01$. * $P < 0·001$.

sugar-beet experiments plotted on a scale relative to the value of E for $L = 1$ (Fig. 2*b*) were almost identical. These results show that change in L had nearly the same proportional effect on E whatever the absolute magnitude of E, except that for kale the effect appeared to be smaller early in the season than later.

If the relation between E and L is of the form $E = a+bL$ then

$$EL = aL+bL^2. \tag{1}$$

The crop growth-rate C ($= EL$) should therefore increase less and less rapidly as L increases, reaching a maximum when $L = -(a/2b)$ (where $a = \bar{E}-b\bar{L}$), and subsequently decreasing with further increase in L. The values of C given in Table II appear to show such a curved relation of C to L, and in Exps. 4, 5, and 6 maximum values of C occurred within the experimental range of L. Regressions of C on L of the form

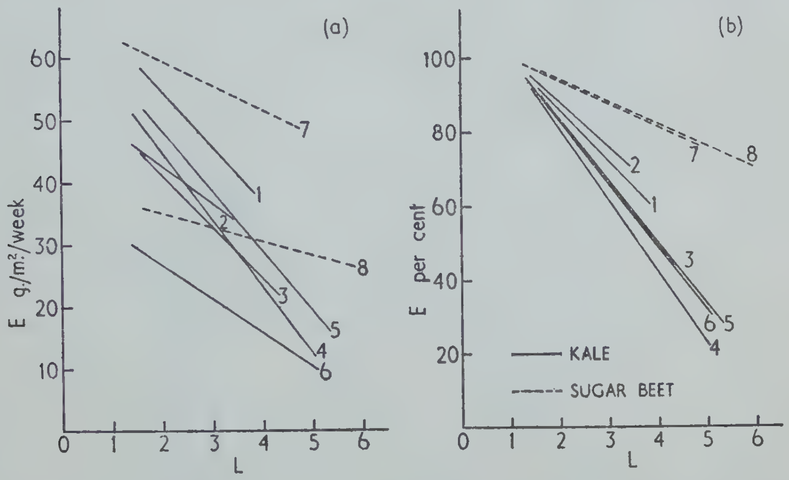

FIG. 2. Linear regression lines of net assimilation rate (E) on mean leaf-area index during the experimental period (L), (*a*) when E is expressed in absolute units, and (*b*) when E is expressed as per cent. of its value for $L = 1$. The numbers attached to the lines refer to the experiments listed in Table I.

$$(C-\bar{C}) = b_1(L-\bar{L})+b_2(L^2-\overline{L^2}) \tag{2}$$

were fitted to the treatment mean of C in each experiment. This curve differs from (1) in that it is not constrained to pass through the origin. Only in Exp. 4 were both coefficients b_1 and b_2 significant (Table IV), but there were significant linear regressions of C on L in Exps. 1 and 2 on kale, and 7 and 8 on sugar-beet. The maximum value of C on the regression line given by (2) occurs when $L = (-b_1/2b_2)$. Values of L for maximal C calculated in this way or from the coefficients of the regressions of E on L for all the kale experiments (Table IV), were consistent in spite of the high errors of the regression coefficients. The two methods of calculation gave identical results

TABLE IV

Coefficients of Linear and Quadratic Regressions of C on L; the Values of L for Maximal C and the Maximal Values of C Estimated from the Quadratic Regressions

		Kale						Sugar-beet	
Exp.		1	2	3	4	5	6	7	8
Linear regression coefficient[1]		25±5*	25±5*	10±5	−5±5	0±8	3±10	40±5*	21±8†
Coefficients of quadratic regression[2]	linear b_1	60±40	74±40	21±40	78±32†	74±50	40±62	95±31‡	51±46
	quadratic b_2	−6±7	−10±8	−2±7	−13±2†	−11±7	−9±15	−12±7	−5±8
L for maximal C	3	4·6	3·7	5·4	3·0	3·5	3·4	5·1	6·4
	4	4·1	4·6	3·5	3·1	3·5	3·4	8·7	8·9
Maximal C[3]		152	115	102	107	119	67	215	147

† $P < 0·05$. ‡ $P < 0·01$. * $P < 0·001$.

[1] The coefficient b of the regression equation $(C-\bar{C}) = b(L-\bar{L})$.

[2] The coefficients b_1 and b_2 of the regression equation $(C-\bar{C}) = b_1(L-\bar{L})+b_2(L^2-\overline{L^2})$.

[3] Calculated from the coefficients of the quadratic regression.

[4] Calculated from regression of E on L (see text and Table III).

for Exps. 4, 5, and 6; for these, maximal *C* occurred when *L* was between 3 and 3·5. Exps. 1 and 2 gave higher values that varied with the method of calculation, presumably because determination of the maxima involved extrapolation beyond the experimental range of *L*. The same is true of Exps. 7 and 8 on sugar-beet, but the maxima obviously occurred at much higher values of *L* than for kale, in the region of 5 or 6, or possibly even higher still. Though little reliance can be placed on the maximal values of *C* (Table IV) calculated from equation 2, for kale they were consistently a little more than 100 g./m.^{2}/week. Much higher maximal rates are evidently possible with sugar-beet.

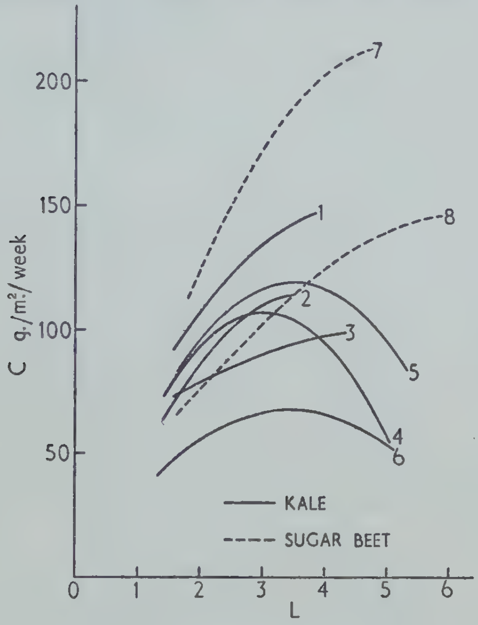

FIG. 3. Quadratic regression lines of crop growth-rate (*C*) on mean leaf-area index (*L*). The numbers attached to the lines refer to the experiments listed in Table I.

The regression lines for the kale experiments plotted in Fig. 3 show that, though the precise location of the maximum may be doubtful, high values of *C* occur when *L* lies between about 3 and 4. In this region *C* is not much affected by variation in *L*, but at higher or lower values of *L* it decreases rapidly. The curves for sugar-beet (Fig. 3) show that maximal *C* was not reached within the range of *L* tested, but their trend indicates that increase of *L* beyond about 6 is unlikely to cause further increase in *C*.

DISCUSSION

The experiments on kale showed that net assimilation rate decreased with increase in leaf-area index, but they were not accurate enough to establish the precise form of the relation of *E* to *L*. Over the range of *L* from 1 to 5,

E apparently decreased at a nearly uniform rate, and the values of E plotted against L were fairly well fitted by straight lines. There was no indication of any region in the lower range of L where E was independent of L, and if such a region exists, as seems likely *a priori*, it must lie at low values of L below unity. The results for sugar-beet were inconclusive, but the consistency of the two experiments leaves little doubt that E for this crop also decreased with L, but at a much lower rate than for kale. Thus, E of kale was approximately halved by increase in L from 1 to 4 (Fig. 2*b*), but a similar change in L for sugar-beet reduced E by only 15 per cent. Taken at their face value, the results of both sugar-beet experiments (Table II) suggest that there was no decrease in E until L rose above about 3, but this requires confirmation.

Evidence that mutual shading of leaves can account for the decrease of E with increase of L is given by changes induced in other growth attributes by the thinning treatments. Blackman and Wilson (1954) found in shading experiments with *Helianthus annuus* that plants transferred from a low to a higher light intensity had a smaller leaf-area ratio after 4 or 8 days than plants held throughout in the low-light intensity. The ratio of leaf area to leaf weight also was decreased by increase in light intensity. In two of their experiments the relative leaf growth-rate was little affected by transfer from low to higher light intensity, and in a third experiment it was increased. Table V shows that thinning the crop caused a decrease in leaf-area ratio in three of the kale experiments and both sugar-beet experiments, and decreased the ratio of leaf area to leaf weight in all but Exp. 6. It increased the relative leaf growth-rate of kale but had no effect on that of sugar-beet. These changes are what would be expected if removal of part of the crop increases the illumination of the leaves of the remaining plants.

The difference between kale and sugar-beet in the sensitivity of E to change in L is perhaps a consequence of the difference in form of the two plants. The leaves of kale are borne along a tall stem, and so are distributed through a much greater height than those of sugar-beet, which are produced close to the ground on an axis that does not elongate, and this may cause the leaf area of a sugar-beet crop to be more uniformly illuminated than that of a kale crop, especially when L is large. Increase of L may cause a general reduction in light intensity over the whole leaf surface of the sugar-beet crop, while for kale its main effect may be to increase the fraction of the leaf surface that receives light of low intensity near or below the compensation point, without much affecting the illumination of the rest. This explanation implies that a short crop with leaves produced in a narrow zone close to the ground is more efficient in utilizing sunlight in photosynthesis than a tall crop with leaves distributed over a wide range of height above the ground, because a smaller fraction of its leaf area is exposed to light intensities below the compensation point or above saturation, i.e. radiation is distributed more efficiently over the leaf surface, and that the advantage of the short crop increases with increase in L. The only evidence from the present experiments to support this is that E decreased less rapidly with increase in L in Exps. 1

and 2 than in the experiments later in the season (Fig. 2*b*); the younger and shorter kale crop behaved more like sugar-beet in respect of the relation of *E* to *L* than did the older, taller kale crops. Measurements of the variation of light intensity and the distribution of leaf area with height in kale and sugar-beet crops of varied *L* are obviously needed to test this hypothesis.

TABLE V

Treatment Means of Leaf-area Ratio (L/W), Leaf Area : Leaf-weight Ratio (L/w) sq. cm. per g., at the End of the Experimental Period, and of Relative Leaf Growth-rate per cent. per Week (RLGR)

Exp.	Treatment (fraction of plants removed)	L/W	L/w	RLGR
		Kale		
1	2/3	67	168	28
	1/3	63	179	17
	0	65	195	11
	S.E.	1·6	1·6	1·9
2	2/3	77	159	22
	1/3	78	173	18
	0	78	189	12
	S.E.	1·5	2·7	2·6
3	2/3	57	157	18
	1/3	58	172	6
	0	60	189	5
	S.E.	1·8	4·2	2·3
4	3/4	53	160	22
	1/2	51	178	19
	1/4	54	182	12
	0	59	191	13
	S.E.	1·7	2·2	4·2
5	3/4	46	161	15
	1/2	48	173	12
	1/4	47	181	5
	0	50	192	2
	S.E.	1·2	2·5	2·3
6	3/4	31	157	7
	1/2	36	153	2
	1/4	29	160	−4
	0	32	157	−2
	S.E.	1·8	3·8	4·9
		Sugar-beet		
7	3/4	52	167	10
	1/2	54	175	19
	1/4	63	204	21
	0	65	214	20
	S.E.	1·6	4·8	3·8
8	3/4	49	184	9
	1/2	50	190	8
	1/4	52	202	8
	0	54	209	9
	S.E.	1·4	5·0	3·4

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One implication of the results is that differences between species in net assimilation rate must depend on leaf-area index. For example, Exps. 5 and 7, which overlapped in time and so provide a comparison of kale and sugar-beet in similar external conditions, show that E of sugar-beet was little greater than that of kale for values of L near 1, but was nearly three times that of kale when L was about 5 (Table II).

The results also suggest that the inverse correlation between E and L found in comparisons between commercial strains of sugar-beet or varieties of potato (Watson, 1947) is merely an example of a general relation similar to that observed in the present experiments with varied populations of uniform plants of one variety, and may not have a specific genetical basis. However, Fig. 4 shows that the rate of decrease of E with increase in L for five varieties of potato compared in the same experiment was much steeper even than in the kale experiments (Fig. 2). The coefficient of the regression line in Fig. 4, $-47 \pm 8{\cdot}9$, shows that unit increase in L within the groups of potato varieties was associated with a decrease in E amounting to 43 per cent. of the value of E for $L = 1$ (estimated by extrapolation of the regression line); the largest corresponding rate of decrease in the kale experiments was only 19 per cent. (Table III, Exp. 4). It seems probable, therefore, that if the five varieties were compared at constant L, part of the variation between them in E might still persist. Increase in L within the group of varieties was a consequence of larger leaves and not of more leaves (Watson, 1947),

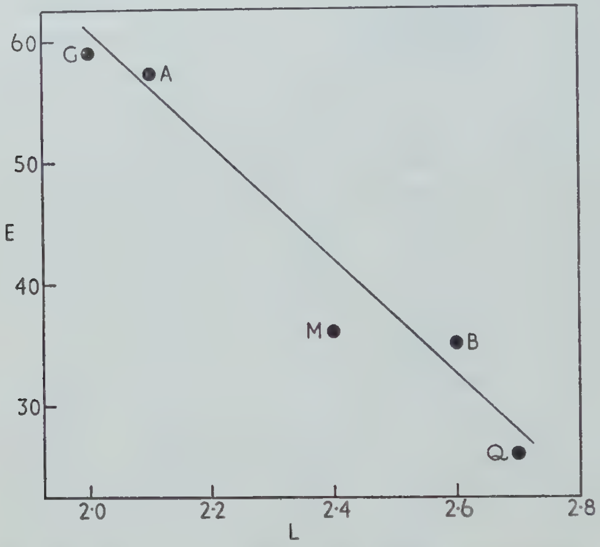

FIG. 4. Net assimilation rate (E), g./m.^{2}/week, plotted against leaf-area index for five varieties of potato; means of two fortnightly periods, 11 August–8 September, 1938, near to the time of maximum leaf area. Varieties: G = Great Scot, A = Ally, M = Majestic, B = Arran Banner, Q = British Queen. (Data from results of Exp. 10 of Watson, 1947.)

and the possibility remains that the varieties with larger leaves may have had a lower photosynthetic efficiency due to internal factors of structure or composition, independently of the effect of differences in L that resulted from variation in leaf size.

As E of kale is so greatly influenced by change in L, increase in L of a kale crop with advancing age would be expected to cause a decline in E independently of seasonal climatic change, or of any effect of age on internal factors. For sugar-beet, the effect on E of increase in L and the consequent ontogenetic drift of E should be much smaller than for kale. Experiments on sugar-beet with varied date of sowing (Watson, 1947) have shown only a small, barely detectable, decline in E with age, but no comparable work has been done on kale.

Similarly, increase in L induced by fertilizer application to a field crop presumably causes a corresponding decrease in E. This might be sufficient to offset or greatly reduce any specific beneficial effect of increased nutrient supply on photosynthetic efficiency, and differences in the relation of E to L like that between kale and sugar-beet could cause variation between species in the response of E to varied nutrition.

The maximum crop growth-rate of kale occurred well within the range of leaf-area index tested. The position of the maximum of C for sugar-beet was located only very roughly; it probably lies between $L = 6$, the upper limit of the experimental range, and $L = 9$. Although 6 is about the highest value of L found for sugar-beet crops at Rothamsted, higher values undoubtedly occur on more fertile soils, e.g. in the Fens, though they have not been measured, so it is unlikely that the yield of sugar-beet could be much increased beyond the current agricultural range by increase in L, especially as C near its maximum will change only slowly with change of L. The leaf-area index of heavy kale crops is already far in excess of the optimum for dry-matter production; in the present experiments this was true from July onwards. Consequently, there would be no advantage in still further increase of L; on the contrary, the total yield of dry matter per unit area of land under a kale crop might be increased if L were decreased in the later stages of growth and held near the optimum by repeated thinning of the crop or partial defoliation. Evidently the main opportunity for increasing dry-matter yields of sugar-beet or kale, beyond the highest attainable at present, lies in the early stages of growth when L is far below the optimum. The particular importance of maintaining high L throughout the months of June and July, when conditions are most favourable for photosynthesis, has already been emphasized (Watson, 1947).

Kasanaga and Monsi (1954) have attempted to estimate the optimal leaf-area index for dry-matter production by calculations based on a simplified model of the height distribution of leaves in a plant community, on the light transmission of leaves, and on curves relating rate of photosynthesis to light intensity for single leaves. Making many assumptions, including some of doubtful validity, they found that optimal L in full daylight was 3 for a community composed of only sun leaves, and 5 for a mixed community of sun and shade leaves. These estimates agree with the values found experimentally for kale.

If the decrease of net assimilation rate with increase of leaf-area index were the result not of shading but of mutual interference between leaves in their supply of CO_2, the rate of dry-matter production per unit area of land should be CO_2-limited when L is large, and so the upper limit to crop growth-rate should be set by the rate at which CO_2 can move into the crop from the atmosphere above it. Maximal C should then be the same for all crops, and C should not fall from its maximum with further increase of L. However, comparisons between Exps. 5 and 7 and between Exps. 6 and 8 show that maximal C of kale was much less than that of sugar-beet in comparable conditions. This agreed with the interpretation that E decreases with increase in L through mutual shading of the leaves, and that the form of the curve relating C to L and the maximal value of C are determined by the way in which the spatial distribution of leaves affects the utilization of incident light. It is true that differences in maximal C between species could arise by differential respiratory loss during the hours of darkness, even if the total CO_2 fixation per unit area of crop during the daylight hours were constant, but the difference between kale and sugar-beet seems too large to be accounted for in this way.

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Sexuality in Diploid and Hexaploid Races of *Mercurialis annua* L

BY

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With six figures in the text.

ABSTRACT

The variation in sex expression found between individuals of *Mercurialis annua* agg. is described, distinction being made between 'regular' and 'irregular' monoecism. There is shown to be a relationship between sexuality, morphology, and chromosome number.

M. annua sensu stricta and *M. annua* var. *ambigua* are shown to be cytologically distinct, the former being diploid, the latter hexaploid. It is recommended that *M. annua* var. *ambigua* be regarded as a species—*M. ambigua*—as originally proposed by Linnaeus *fils*.

Past investigations into the inheritance of sex are reviewed and results of further investigations into the inheritance of monoecism in *M. annua* var. *ambigua* are presented. Evidence obtained supports the hypothesis that the allosome constitution of male plants is *XXXXXY* and that of monoecious plants is *XXXXXX*.

INTRODUCTION

IN 1762, Linnaeus *fils* in his 'Plantarum rariorum Horti Upsaliensis Decas I' separated a new species, *Mercurialis ambigua*, from *Mercurialis annua* L. It was described as monoecious instead of dioecious, and had a more southerly distribution in Europe. Since that time doubts have arisen as to whether this monoecious race justifies the rank of a taxonomic species, and it has been redescribed under *M. annua* as var. *ambigua* Duby and as var. *monoica* Moris. These difficulties in classification have arisen because, although the true monoecious race is very distinct, numerous grades of monoecism exist within *M. annua*. The present paper reports the results of investigations into the morphology, cytology, and genetics of the annual forms of *Mercurialis*, and provides evidence as to the true status of *M. ambigua*.

THE DIVERSITY OF SEX EXPRESSION

Yampolsky, C. and H. (1922) have described a number of the more common sexual forms found in annual *Mercurialis*, and this terminology is adopted in the present account.

Normal sex types

(*a*) Dioecism. This is the condition in *M. annua* sensu stricta; male and female flowers are borne on different individuals, and characteristically in different types of inflorescence (Figs. 1*a* and 1*b*).

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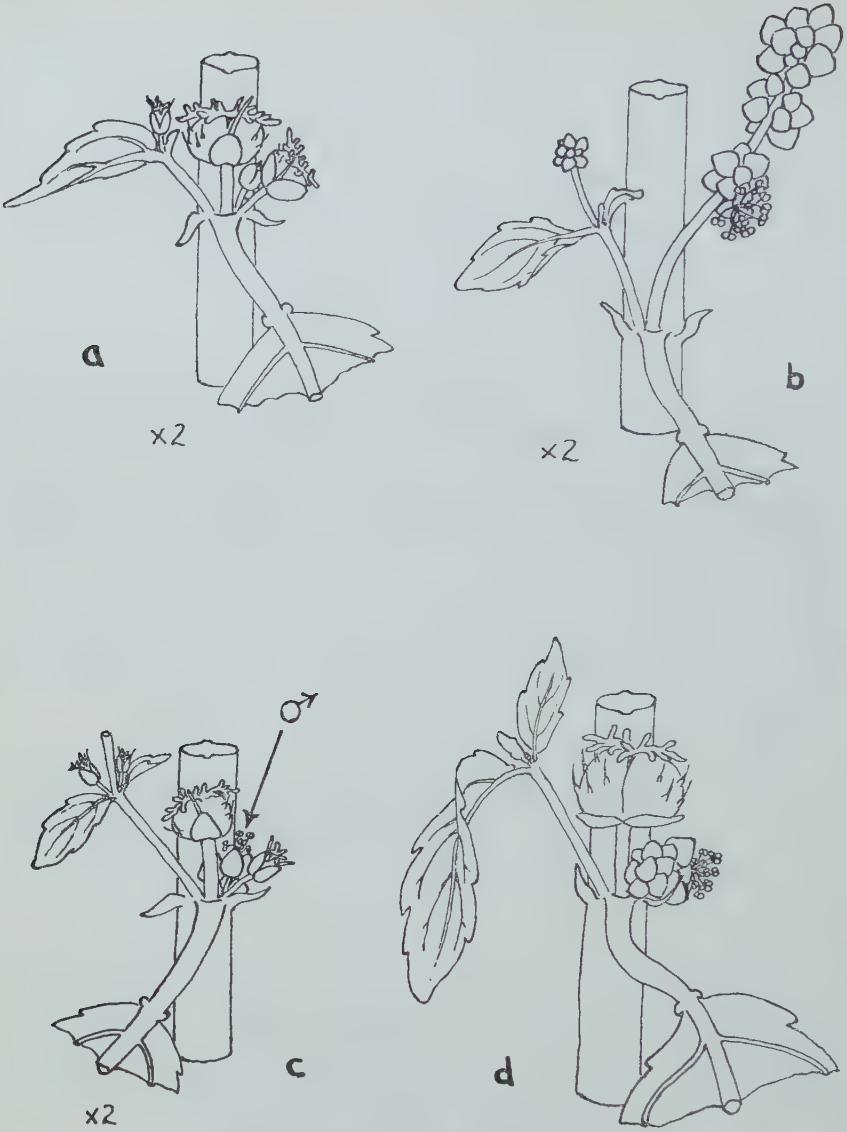

Fig. 1. *Inflorescence types in Mercurialis*

(*a*) Normal diploid female *M. annua*; (*b*) normal diploid male *M. annua*; (*c*) upper node of an irregularly monoecious tetraploid hybrid; (*d*) regularly monoecious hexaploid *M. ambigua*

(*b*) Monoecism. *M. ambigua* is normally said to be monoecious, but although its populations include a majority of monoecious individuals, some are invariably wholly male. It would be convenient to define this condition as andromonoecism but Yampolsky used this term to denote a plant bearing hermaphrodite and male flowers. In the monoecious plants there is a single female flower and a lateral group of male flowers in each leaf axil (Fig. 1*d*).

Abnormal sex types in M. annua

(*c*) Hermaphroditism. Although Yampolsky (1919) has illustrated flowers which have the appearance of being hermaphrodite, true hermaphroditism probably does not occur in *M. annua*. Reynier (1922) described the female flowers as 'semihermaphrodite', quoting the description given by Laurent de Jussieu in his *Genera Plantarum* of 1789, 'Flores feminei: *Filamenta* 2 brevia, sterilia, ex imis sulcis enata et appressa germini.' The structures which he considered as filaments in the female flowers have never been observed to bear anthers, and the opinion of Weiss (1906) and Bitter (1909) that they are nectaries is probably nearer the truth. Neither male nor female flowers develop any rudiment of organs of the opposite sex (Figs. 2*e* and 2*f*).

(*d*) Irregular monoecism. Irregular monoecism may be defined as the occasional production of flowers of the opposite sex on an otherwise normal male or female plant, and is not to be confused with the regular monoecism of *M. ambigua*. Bouche (1881) and Bitter (1909) both noted a strong tendency for male flowers to develop on female plants; the tendency for the production of female flowers on male plants is much weaker, and the phenomenon has not been observed by the present author. A male inflorescence bearing a female flower is illustrated by de Vries (1903).

Several workers have observed that female plants tend to become irregularly monoecious at the end of their growing season. Yampolsky (1919, 1920*a*) reported on the behaviour of foplants ur which produced only female flowers throughout the summer, but which developed some male and intersexual flowers from the end of August onwards. Blaringhem (1922) examined wild populations growing at Plessis-Macé in France and found similar plants which were female throughout the summer but which later produced intersexual and finally male flowers in their upper leaf axils.

Two types of irregular monoecism were described by Gillot (1924*a*) from French populations at Magneux. The first occurred in plants which were predominantly female, but which produced some male flowers in the upper axils at the end of the growing season. This is apparently the same phenomenon observed by Yampolsky and Blaringhem, and an upper node of a plant of this type is illustrated in Fig. 1*c*. Gillot's second type was among male plants, some of which bore occasional female flowers on otherwise entirely male inflorescences. A few regularly monoecious plants of *M. ambigua* have been found which, at some stages of development, resemble his illustrations, so that it is possible that these 'male' plants were in fact regularly monoecious individuals growing in an environment not conducive to the expression of femaleness.

The terms 'regular' and 'irregular' are proposed to avoid confusion between the two types of monoecism. Regularly monoecious plants never produce more than one female flower per leaf axil and occasionally develop only male flowers; irregularly monoecious plants, on the other hand, always have several female and very few, or no, male flowers per leaf axil.

(*e*) Intersexuality. This is best described as the development of organs of intermediate sex in individual flowers and is not to be confused with hermaphroditism. The range of such aberrations is very wide (Molliard, 1898; Yampolsky, 1920*b*; Blaringhem, 1922). Female flowers may be found which possess one or two normally developed stamens; almost pure male flowers occur sometimes in which some of the stamens are tipped with stigmas, and female flowers occasionally develop pollen sacs on the carpel walls.

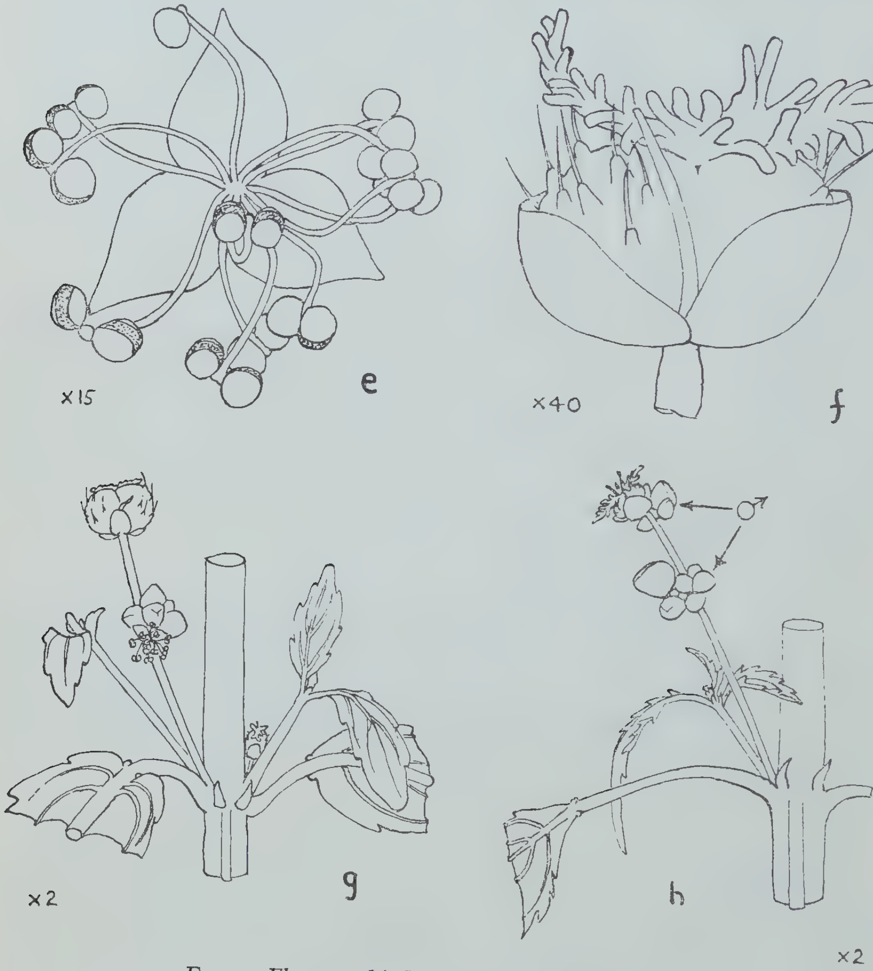

FIG. 2. *Flower and inflorescence types in Mercurialis*

(*e*) Single pure sexed male flower of *M. annua*; (*f*) single pure sexed female flower of *M. annua*; (*g*) and (*h*) Atypical inflorescence forms found occasionally on regularly monoecious hexaploid *M. ambigua.*

(*f*) Male sterility. Three male plants grown by the writer during 1954 (two from Coimbra seed, one from Liège seed) were completely sterile, and many of the monoecious plants grown from Coimbra seed (see below) produced sterile male flowers, although the female flowers borne by them set seed freely. The stamens in the sterile flowers produce no pollen, and the flowers are often caducous.

THE RELATIONSHIP BETWEEN THE INFLORESCENCE OF *MERCURIALIS* AND THE EUPHORBIACEOUS CYATHIUM

The cyathium is an extremely specialized form of inflorescence peculiar to the Euphorbiaceae in which the essential organs of the component flowers are disposed in such a manner that the whole resembles a single flower—a 'pseudanthium'. The inflorescence of *Mercurialis*, whilst superficially so unlike a cyathium, shares certain important features in common with it. The main forms of the Mercurialis inflorescence are:

(*a*) The normal male inflorescence (Fig. 1*b*). This consists of several clusters of male flowers on a long pedunculate spike.

(*b*) The normal regularly monoecious inflorescence (Fig. 1*d*). At each node of regularly monoecious plants there is a central female flower on a peduncle in the leaf axil and a lateral group of male flowers. Under certain environmental conditions the situation illustrated in Fig. 2*g* occurs, where the female flower and the lateral group of male flowers are borne on an elongated peduncle. Occasionally the form shown in Fig. 2*h* is found; this is very similar to the normal male inflorescence but for the terminal female flower.

(*c*) The normal female inflorescence (Fig. 1*a*). The first flower to develop is in the same position as the single female flower of the monoecious inflorescence, but in place of the lateral cluster of male flowers a few lateral female flowers develop.

The basic pattern of inflorescence can thus be regarded as a female flower terminating a peduncle which bears clusters of males. In Fig. 3 this basic type is compared with the others found. The similarity between the inflorescence of *M. ambigua* and the cyathium can be seen from the diagrams of Fig. 4. In each there is a central female flower with lateral groups of males below it. The normal monoecious inflorescence of *M. ambigua* consists of a female flower and only one group of males, whereas in the cyathium there are five groups. However, the abnormal inflorescences of *M. ambigua* (Figs. 3*c*, 4*b*) are more similar to the cyathium, providing a link which relates the normal monoecious inflorescence, and indeed the condition found in the dioecious *M. annua* sensu stricta, to the cyathium. The hypothetical 'basic type' of *Mercurialis* inflorescence (Fig. 3*b*), with more clusters of male flowers, fits even more closely.

CYTOLOGY

Chromosome numbers

All previous reports concerning chromosome numbers have referred to the dioecious species, *M. annua*. Strasburger (1910) and Yampolsky (1925, 1933)

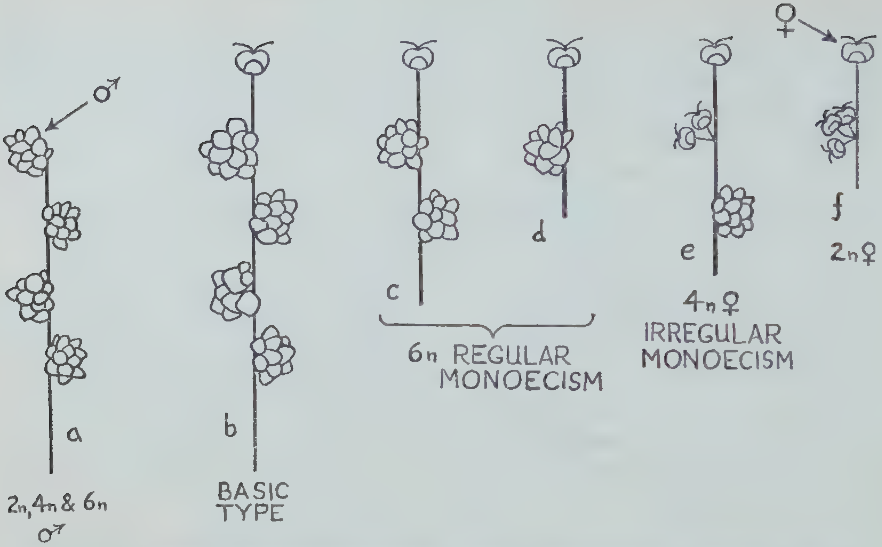

Fig. 3. Range of inflorescence structure found in *Mercurialis* compared with a hypothetical 'basic type'

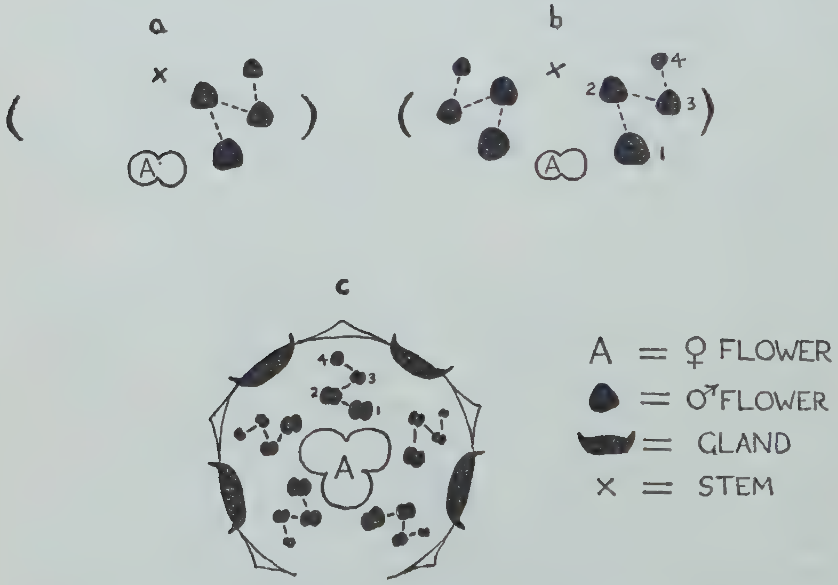

Fig. 4. Euphorbiaceous inflorescence diagrams; (*a*) and (*b*) inflorescence types found in *Mercurialis ambigua*, (*c*) inflorescence of Euphorbia peplis (after Engler and Prantl)

found that in this race $2n = 16$. The same somatic chromosome number has been observed in three plants grown from seed received from Mainz, Germany, and in twenty plants from a wild population in London. Seeds obtained from Liège produced plants of several different sex types, but all normal male and female plants were also found to be diploid, with $2n = 16$ (Fig. 5*a*). Metaphase plates of meiosis II showing the haploid chromosome number of 8 in this strain are illustrated in Fig. 6*a*.

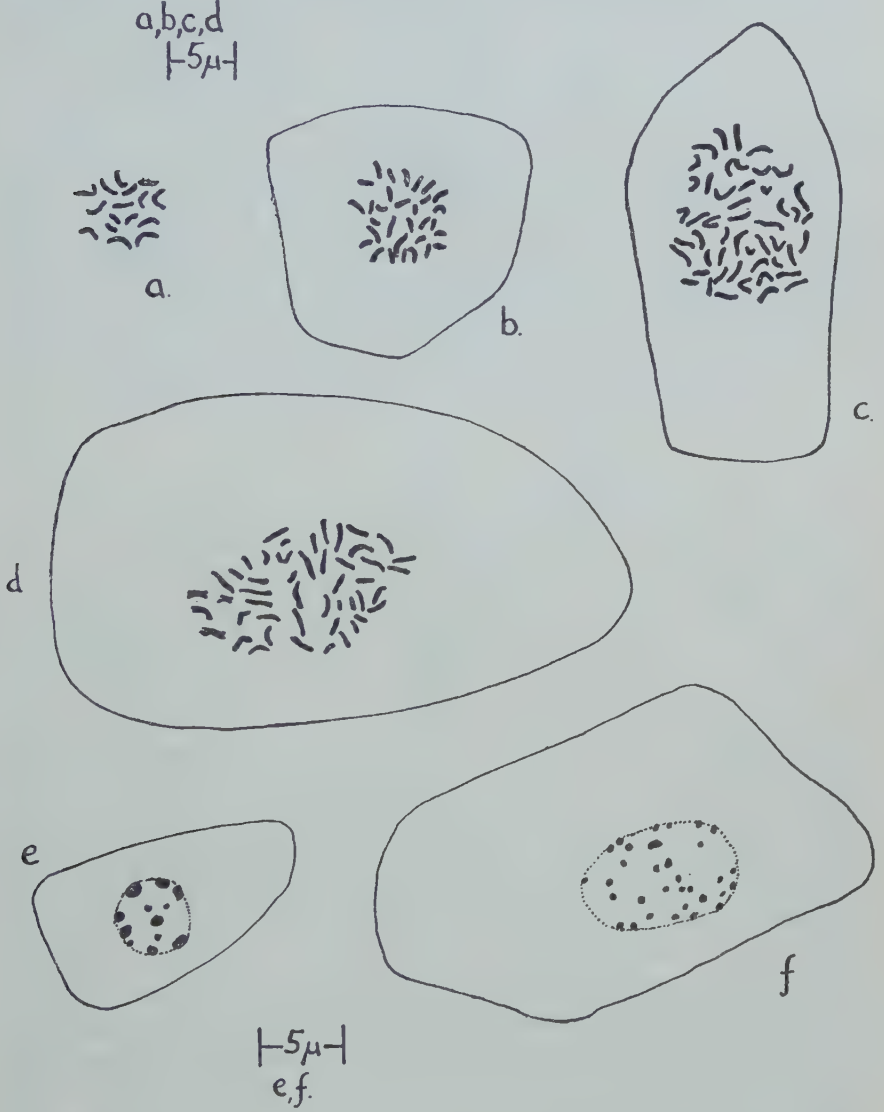

FIG. 5. Isolated cells from root tip squashes: (*a*) to (*d*) showing metaphase plates: (*a*) *M. annua*, (*b*) tetraploid hybrid (*M. annua* × *M. ambigua*), (*c*) and (*d*) *M. ambigua*. (*e*) and (*f*) interphase nuclei showing chromocentres; (*e*) *M. annua*, (*f*) *M. ambigua*

Seeds obtained from Coimbra, Portugal, produced some male and some monoecious plants, and all in which chromosome counts were made (7 male, 12 monoecious) were found to be hexaploid, $2n = 48$, or approximately so (Figs. 5*c* and *d*). Stages of meiosis in pollen mother cells from plants from this source are illustrated in Figs. 6*b* and *c*, showing the reduced chromosome number of 24.

Mercurialis possesses chromocentres which are visible on staining in all resting nuclei. Yampolsky (1937) states that there is no correlation between chromosome number and the numbers of such chromocentres in diploid individuals, but nevertheless the difference between diploid and hexaploid is very striking (Figs. 5*e* and *f*), and this can be made an immediate test to distinguish between them.

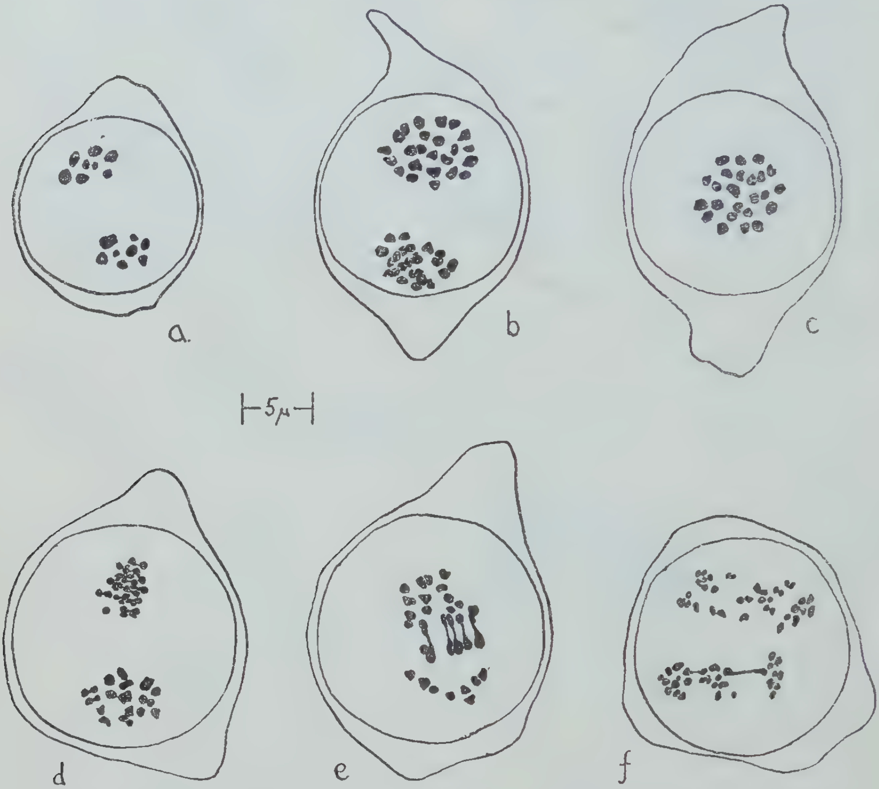

FIG. 6. Meiotic divisions of pollen mother cells. (*a*) metaphase of meiosis II in *M. annua*; (*b*) and (*c*) similar stages in *M. ambigua*; (*d*) anaphase of meiosis II in tetraploid hybrids; (*e*) and (*f*) similar stages in tetraploid hybrids showing irregular division and chromosome bridges

It was of interest to establish the chromosome numbers of plants obtained from Liège seeds since these were so diverse in sex type. In 15 monoecious individuals a somatic chromosome number of $2n = 48$ was found, and in a sample of 16 pure-sexed females and 11 pure-sexed males the number proved to be $2n = 16$. No hexaploid males were found. The monoecious Liège plants were exactly similar to the regular monoecious plants of *M. ambigua.* It appears that the seed from Liège, which was collected from market gardens in the environs of the city, was derived from two distinct races, and that none was of hybrid origin.

Crosses were made between diploid *M. annua* and hexaploid *M. ambigua*, and, as expected, examination of root-tip mitosis and pollen meiosis showed hybrids to be tetraploid, $2n = 32$ (Figs. 5*b*, 6*d*, *e*, and *f*). The pollen fertility of male hybrids was estimated by staining with acetocarmine. In one plant there were 4 fertile grains out of 195 examined, but the pollen was completely infertile in other plants observed. Divisions in the pollen mother cells were found to be very irregular (Fig. 6*e*). In no case did a female plant of hybrid origin set seed, even when the flowers were liberally pollinated with fertile grains from diploid and hexaploid plants. The hybrid *M. annua* × *M. ambigua* must therefore be regarded as almost entirely sterile.

The somatic chromosome number of all plants from Liège and Coimbra examined is shown in Table I. Of the twenty-seven monoecious plants studied all were hexaploid, which suggests that regular monoecism does not occur in diploid plants. The significance of this will be discussed later.

TABLE I

Somatic Chromosome Numbers in Plants of Mercurialis

Chromosome no.	$2n = 48$			$2n = 16$		
Seed source	Coimbra	Liège	Total	Coimbra	Liège	Total
No. of Female plants . .	—	—	0	—	16	16
No. of Male plants . .	7	—	7	—	11	11
No. of Monoecious plants .	12	15	27	—	—	0

Sex chromosomes

The genetical evidence suggests that the male is heterogametic, but sex chromosomes have not been distinguished in *M. annua* by previous workers including Strasburger (1910), Malte (1910), and Yampolsky (1925), nor have they been observed in the present work.

Sinoto (1929) and others have observed bimodal distributions of pollen grain size in dioecious plants in which the male is heterogametic, although this is not so in all; thus D. Löve (1940) and Westergaard (1940) found no differences in pollen grain size in *Melandrium*, notwithstanding the earlier report of Greguss (1927) to this effect, and A. Löve (1944) found the pollen diameter of *Rumex acetosa* was distributed unimodally even although the male-determining grains have 8 and the female-determining 7 chromosomes.

Pollen-grain size in *M. annua* shows a unimodal distribution. The diameters of 1,195 grains were measured and found to be distributed normally with mean $21{\cdot}19\mu$ and standard deviation $1{\cdot}061$, but there was no sign of bimodality. It is conceivable, however, that with a magnification very much greater than × 1,500, and therefore smaller class intervals, bimodality might yet be demonstrated.

The relationship of morphology and chromosome number

A few characters were studied biometrically in the diploids and hexaploids, and significant differences observed.

(*a*) Seed length and breadth. Seeds were collected from diploid and hexaploid plants grown under natural daylight during the summer, and also from diploid females which had been crossed with hexaploid monoecious plants. Seeds from the latter source would, of course, be expected to contain tetraploid embryos. The measurements obtained from samples of fifty or more are recorded in Table II. Although the differences observed are not large, all are statistically significant except between breadth of tetraploid and hexaploid seeds.

(*b*) Stomatal size. The number of stomata in a microscope field of 0·36 mm. diameter was counted on seven diploid and seven hexaploid plants growing together in continuous light. In each case stomata were counted on similar regions of the lower epidermis of fully expanded leaves at the fifth node. Five to thirteen counts were made on each leaf, and greater numbers where the variation in number per field was high. The differences between diploids and hexaploids are statistically significant, and correspond well to those expected on the assumption that cell volume is doubled by doubling the volume of chromatin. Similar counts on a few tetraploid plants from the cross diploid-female × hexaploid-monoecious gave results not significantly different from those from hexaploid plants.

(*c*) Plant height. Seeds obtained from Liège were sown in different daylength conditions, some receiving natural days of about 16 hours illumination, and others in artificially shortened days of 8 hours. Three types of plant were obtained from this Liège seed, male, female, and monoecious, and after 8 weeks' growth the heights were measured under each daylength condition. The results are recorded in Table IV.

The heights of diploid and hexaploid (monoecious) plants in short days do not differ significantly between themselves or from heights of diploid plants in long days. The hexaploid monoecious plants in long days, however, differ very significantly from all of these groups. Even although only three appeared in the short-day series, a *t*-test shows the difference between heights in short and long days to be significant at the 1 per cent. level of probability.

In another experiment seven hexaploid monoecious plants from Coimbra seed, six diploid plants from London seed, and seven tetraploids resulting from a cross between the Coimbra and London races were grown together in 16-hour days at a constant temperature of 21° C. The average height (excluding the hypocotyl) of these plants after 45 days' growth was: hexaploids 11·55 cm., tetraploids 11·18 cm., diploids 1·9 cm. In each character measured, tetraploids differ little from hexaploids.

The measurements indicate that the difference between diploid and hexaploid plants is much greater when the daily periods of illumination are longer as a result of the greater influence of daylength on hexaploids, and suggest that the differences may sometimes only be revealed under particular environmental conditions.

TABLE II

Seed Dimensions in Different Mercurialis *Cytotypes*

(Standard errors shown in brackets)

Cytotype	*2n*	*4n*	*6n*
Seed length	1·97 mm. (0·01)	2·15 mm. (0·01)	2·33 mm. (0·01)
Seed breadth	1·40 mm. (0·01)	1·58 mm. (0·01)	1·57 mm. (0·01)
No. of seeds measured	76	50	62

TABLE III

Stomatal Distribution on Leaves of Different Mercurialis *Cytotypes. Numbers per field of 0·36 mm. diameter on individual plants*

(Standard errors shown in brackets)

Cytotype	*2n*		*4n*	*6n*	
Sex	Male	Female	Female	Male	Monoecious
Number of stomata per field	17·8 (0·86)	10·2 (0·66)	9·6 (0·49)	9·8 (0·51)	10·8 (0·52)
		19·5 (0·66)	9·0 (0·56)		7·3 (0·29)
		13·4 (0·61)	6·3 (0·32)		5·8 (0·44)
		17·8 (0·71)			6·6 (0·53)
		15·8 (0·86)			6·4 (0·49)
		22·3 (1·05)			7·3 (0·60)
					8·5 (0·61)
Average	17·8	16·5	8·3	9·8	7·5

TABLE IV

Stature of Plants of the two Mercurialis *Cytotypes under Different Daylengths*

(Standard errors shown in brackets)

	No. measured	Height (cm.)	No. measured	Height (cm.)
2n male	7	22·6 (1·00)	7	25·3 (1·00)
2n female	12	25·4 (1·26)	16	28·6 (1·11)
6n monoecious	3	24·3 (1·71)	7	43·9 (3·13)

THE INHERITANCE OF SEX

Dioecism

Among the earliest breeding experiments concerned with sex in *Mercurialis* are those of Krüger (1908) and Bitter (1909). Krüger isolated female plants and found that they apparently set seed in the absence of pollen; since the offspring were all female, he concluded that parthenogenesis had occurred. Bitter germinated seed from isolated female plants and obtained 723 females and 21 males. He recorded the presence of occasional male flowers on his female plants, and concluded that his results could be explained assuming that pollen from these flowers fertilized the ovules of the isolated plants. The presence of the few male offspring might be accounted for by the fact that the soil in which the seed was sown was not sterilized. Malte (1910) confirmed that seed was only set on isolated female plants following pollination from sporadic male flowers or from other sources.

All authors are agreed that the progeny of female plants pollinated from male flowers borne by female plants is practically exclusively female (results summarized in Table V), and it is clear that the occasional appearance of male offspring cannot be regarded as significant, since, *Mercurialis* being anemophilous, it is extremely difficult to exclude all pollen from foreign sources. It is surprising that Sansome (1938), in the face of so much evidence that the female is the homogametic sex, should state that 'of all species so far examined, only *Mercurialis annua*, in which the female is heterogametic, and *Silene otites* have an excess of males in the wild'.

All experiments in which females have been crossed with males show that the proportion of sexes amongst the offspring is 1 ♂:1 ♀ (see Table V). The results are supported by observations of sex ratios in wild populations. Heyer (1884) found a ratio of 106 ♂♂ : 100 ♀♀ in a population of 21,000 plants, and Correns found 51·2 per cent. males in a population of about the same size.

Experiments in which male plants have either been selfed or crossed with other male plants have not yielded consistent results (Table V). Strasburger (1910) and Yampolsky (1919) obtained only male offspring from seeds collected off male plants; Kuhn (1936, 1939), and Gabe (1939) found that ♂× ♂ produced offspring in the ratio of 3 ♂ : 1 ♀. The conflict between these results is outstanding and the position needs reconsideration bearing in mind the existence of hexaploid monoecious forms.

Experiments on the effects of environment on sex expression of *M. ambigua* (Thomas, 1956) provide a clue to the causes of the inconsistency of breeding results. Production of female flowers is delayed under warm long-day conditions, and it is thus not possible to distinguish between young monoecious and male plants.

Strasburger (1910) had great difficulty in finding male plants which produced female flowers, and the fifteen plants used in his ♂× ♂ experiments were collected from several different sites. He used these plants when they were young; and his description of them exactly fits young regularly monoecious plants under warm long-day conditions. The offspring, which were sexed as soon as it was possible, were apparently all male. However, monoecious plants sexed at this stage of development would have appeared male, and in all probability the so-called males were monoecious and would have produced female flowers later. This interpretation explains the results satisfactorily, as selfed monoecious plants produce nothing but monoecious offspring which appear male initially under certain conditions.

Yampolsky's result is not explained so easily. The seeds from which he grew his male parents were apparently obtained from ♀× ♂ crosses. Of 790 male plants grown only 35 set any seed. There is the possibility that the 35 plants were monoecious with only a slight degree of femaleness, but the evidence for this is very much weaker than in the case of Strasburger's plants.

From the evidence provided by the experiments of Strasburger and Yampolsky the latter built up a theory of cytoplasmic sex inheritance in *Mercurialis*. His evidence is really outweighed by the more recent results of Kuhn (1939) and Gabe (1939) which indicate that the male is heterogametic. Both these workers found that by crossing males offspring were produced in the ratio 3♂:1♀. Of these males, one-third had indehiscent anthers and produced little pollen. This is the result expected if the male plants have XY allosomes. One-quarter of the progeny from the $XY \times XY$ crosses would be YY and these might be almost sterile. Offspring resulting from the cross between females and the almost sterile males were all fertile males, which supports the theory that the sterile males were YY plants.

TABLE V

Offspring Resulting from Breeding Experiments with Mercurialis

Cross	Author	Date	No. of seeds sown	Progeny Female	Progeny Male
♀× ♀	Krüger	1908	?	All	—
♀× ♀	Bitter	1909	?	723	21
♀× ♀	Strasburger	1909	907	148	—
♀× ♀	Yampolsky	1916	1,491	418	—
♀× ♀	Kuhn	1939	?	6,656	12
♀× ♀	Gabe	1939	?	502	3
♀× ♂	Strasburger	1909	?	40	31
♀× ♂	Yampolsky	1919	?	31	36
♀× ♂	Gabe	1939	?	1,507	1,324
♂× ♂	Strasburger	1910	74	—	36
♂× ♂	Yampolsky	1919	283	—	75
♂× ♂	Kuhn	1939	?	176	585
♂× ♂	Gabe	1939	?	179	436

Monoecism

Few investigations into the inheritance of monoecism in *M. ambigua* had been made before those carried out by the writer during 1954 and 1955. Nicolas (1923*a*) and Gillot (1924*a*) found that offspring produced by crossing monoecious plants of *M. ambigua* were all monoecious, but the sex-determining mechanism within this race was not known.

Several possibilities concerning the chromosomal constitution of the hexaploid *M. ambigua* plants can be based on the assumption that in diploid plants males are the heterogametic sex. Four types of plant were found in the stock obtained from Coimbra, and as a working hypothesis it was assumed that their allosome constitutions were as shown below:

Monoecious with sterile male flowers (6*n* Mon. SM) $\equiv XXXXXY$
Monoecious with fertile male flowers (6*n* Mon. FM) $\equiv XXXXYY$
Male plants with fertile male flowers (6*n* F ♂) $\equiv XXXYYY$
Male plants with sterile male flowers (6*n* S ♂) $\equiv XXYYYY$

On this hypothesis it was thought probable that *XXXXXX* plants would be female. Crossing was facilitated by the existence of monoecious plants on which the male flowers were sterile, and these were used as the female parents in two of the first three crosses made to test the above hypothesis. The crosses made were as follows:

	Female parent		Male parent
A.	6*n* Mon. SM	×	6*n* Mon. FM
B.	6*n* Mon. FM	×	6*n* Mon. FM
C.	6*n* Mon. SM	×	6*n* F ♂

The numbers of offspring obtained from these crosses are shown in Table VI together with the numbers of progeny of the various types expected on the above hypothesis.

TABLE VI

Progeny of Crosses between Various Sexual Types of Mercurialis ambigua

Sex		6*n* ♀	6*n* Mon.	SM 6*n*	Mon. FM	6*n* F ♂	6*n* S ♂	Germination
A.	Observed	—	13	—	21	—	—	23%
	Expected	3·4	13·6	—	13·6	3·4	—	—
B.	Observed	—	7	—	32	—	—	8·2%
	Expected	1·5	9·4	—	17·2	9·4	1·5	—
C.	Observed	—	—	176	—	153	1	34%
	Expected	8·2	—	230·2	—	82·7	8·2	—

The results of crosses *A* and *B* agree with those of Nicolas and Gillot; regular monoecious plants, when crossed with other regular monoecious plants, produced only monoecious offspring. These results, as well as those of cross *C*, however, differed significantly from those expected on the basis of the proposed hypothesis.

As a further test, a cross (*D*) was made between diploid females and hexaploid monoecious male-fertile plants and the results are shown in Table VII.

TABLE VII

Progeny of a Cross between Diploid Female Mercurialis annua *and Hexaploid Monoecious Male-fertile* M. ambigua

Sex		♀	Monoecious	6*n* F ♂	Germination
D.	Observed	88	—	—	23%
	Expected	17·6	52·8	17·6	—

It is obvious that this observed result does not fit the original hypothesis.

In all these results the low percentage germination obtained is outstanding. It is unlikely, however, that this would have influenced the sex ratios observed. Gillot (1924*b*, 1925) followed the germination of 4,000 *M. annua* seeds over a period of 4 years, after which time 75 per cent. had germinated, and he found no difference between males and females in the capacity to germinate. The sex ratio obtained in the first year was not significantly different from that observed amongst the plants grown from seeds which did not germinate until 4 years after sowing. It thus seems safe to assume that the sex ratios obtained in the experiments described in this paper approximate closely to those which would occur with 100 per cent. germination.

The results obtained fit the hypothesis that (*a*) all hexaploid regular monoecious forms are $XXXXXX$, (*b*) hexaploid males are $XXXXXY$ with the possible exception of an occasional $XXXXYY$ plant, and (*c*) male sterility is not affected by the number of Y chromosomes present. The results of the four crosses previously described are in agreement with these propositions:

A. Mon. SM ($XXXXXX$) × Mon. FM ($XXXXXX$) gave 34 monoecious ($XXXXXX$) offspring.
B. Mon. FM ($XXXXXX$) × Mon. FM ($XXXXXX$) gave 39 monoecious ($XXXXXX$) offspring.
C. Mon. SM ($XXXXXX$) × $6n$ F ♂ ($XXXXXY$) gave 176 monoecious ($XXXXXX$) and 154 male ($XXXXXY$) offspring.
D. $2n$ ♀ (XX) × $6n$ Mon. FM ($XXXXXX$) gave 88 female offspring.

Two other crosses were made in order to test this hypothesis further; one (*E*) between diploid females ($2n$ ♀) and hexaploid fertile males ($6n$ F ♂), another (*F*) between hexaploid male-fertile monoecious plants (Mon. FM) and diploid fertile males ($2n$ ♂). The progeny obtained from these crosses are shown in Table VIII.

TABLE VIII

Progeny of Crosses between Diploid M. annua *and Hexaploid* M. ambigua

	Sex	♀	♂	Germination
E.	Observed progeny	47	59	16%
	Expected progeny	53	53	—
F.	Observed progeny	154	129	58%
	Expected progeny	141·5	141·5	—

Neither of these results differs significantly from those expected on the hypothesis that hexaploid males possess one Y chromosome and monoecious hexaploids possess no Y chromosomes. In both cases the probability of obtaining deviations from expected which are as great as these, if the expected results be correct, is greater than 1 in 20.

The sexuality of female plants resulting from the crosses *D*, *E*, and *F* between diploid and hexaploid plants differed slightly from that of diploid females. The latter very occasionally produce a few male flowers late in life but the tetraploid females produced quite large numbers of sessile male flowers. There were few of these initially but after several weeks' growth the numbers increased steadily, and the plants fitted the descriptions of the irregularly monoecious forms found by Blaringhem and Gillot (Fig. 1*c*). This production of male flowers is probably genetically controlled to a certain extent, as such large numbers of male flowers have not been observed on diploid females.

Inheritance of maleness

Breeding experiments indicate that the difference between male and monoecious hexaploids is the possession of a single Y chromosome by the former. This situation is similar to that found by A. Löve (1944) in *Rumex acetosella* agg. *R. acetosella* sensu stricta is a naturally occurring hexaploid in which the female allosome complement is $XXXXXX$ and that of the male is $XXXXXY$. Thus either the Y chromosome is male-determining or sex is determined by the ratio of X chromosomes to autosomes (X:A). The ratio X:Y does not affect the sex expression. Experimental evidence indicates that diploid plants with two Y chromosomes are males, and it is therefore more feasible to assume that Y chromosomes determine maleness than that the ratio of X:A controls sexuality.

In point of fact the Y chromosome should be described as female-inhibiting rather than male-determining. The experiments on environmental influences point to the conclusion that monoecious plants without Y chromosomes have the genetic capacity to develop exactly as males, whereas male plants never produce female flowers. Thus the presence of a Y chromosome possibly ensures that a plant is male by inhibiting the formation of female flowers.

PHYSIOLOGY OF SEXUALITY

Under certain environmental conditions, genetically monoecious plants can be induced to flower as males (Thomas, 1956). Plants grown in continuous light at a low temperature (15° C.) form inflorescences of the normal regular monoecious type at each node whereas at a constant temperature of 24° C. with continuous illumination the production of female flowers is delayed until at least the fifth node in most plants. In one experiment, no female flowers had developed by the time the treatment was discontinued, when the thirteenth internode was elongating. On being placed in short days at a low temperature, these plants developed normal monoecious inflorescences.

Using the knowledge gained from these and other experiments on *M. ambigua* and *Cannabis sativa*, a hypothesis can be built up with regard to the possible mechanism by which sex is determined. A plant in the flowering condition will form female flowers as long as it can produce the morphogenetic factors necessary for the formation of female flowers; when the concentration of these factors is too low, the plant will produce male flowers only. Thus in male plants a gene on the Y chromosome must continually inhibit production or action of these factors, so that only male flowers are produced. Diploid female plants produce many female flowers, but towards the end of the growing season the concentration of female-determining factors falls and a few male flowers develop. Monoecious plants possibly produce only enough of these factors to form one female flower in each leaf axil and the remainder of the flowers at each node are consequently male.

THE TAXONOMIC STATUS OF *M. AMBIGUA L. FIL*

During the first quarter of this century *M. ambigua* was mostly considered as a variety of *M. annua*, though it was given species rank by Linnaeus *fils*.

Nicolas (1923*b*), however, was of the opinion that *M. ambigua* was worthy of a rank higher than varietal. He noted that apart from the regularly monoecious condition of the latter it had leaves with larger teeth and hairs than typical *M. annua*.

The position is now worth reconsidering. The plants grown in London as *M. ambigua* were cytologically distinct from *M. annua*, having a somatic number of 48 chromosomes as opposed to 16 in the latter. The race of *M. ambigua* consists of two forms of plants, namely, male and monoecious. Full details will be given in a later paper, but the main points of morphological difference, which vary greatly with the conditions under which the plants are grown, are shown in Table IX. These measurements were made on plants grown in 16-hour days at 21° C.

TABLE IX

Morphological Differences between M. annua *and* M. ambigua (*averages*)

	M. ambigua	*M. annua*
Leaf length/petiole length	(1·5)–2·1–(2·5)	(1·8)–3·8–(4·5)
Hair length	0·4 mm.	0·2 mm.
Serration shape (length/depth)	2·25	4·6
Height after 45 days' growth .	11·5 cm.	2·0 cm.

The type of monoecism found in *M. ambigua* is quite consistent. Seed is set on monoecious plants following pollination by diploid male plants; this seed produces vigorous tetraploid plants which, however, are completely sterile as a result of irregular meiosis. There is thus a sterility barrier between *M. annua* and *M. ambigua* totally precluding gene exchange between the two.

In the light of these differences, Linnaeus *fils* seems to have been well justified in distinguishing *M. ambigua* as a species, and there is every reason to continue treating it as such.

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The Incorporation of C^{14}-labelled Substrates into the Amino-acids of Groundnut Plants (*Arachis hypogaea*)

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With five figures in the text

ABSTRACT

The amino-acid metabolism of groundnut plants has been studied with special reference to γ-methyleneglutamic acid (γ-MGA) and γ-methyleneglutamine (γ-MG), constituents not found in the great majority of plant species.

The sequence in which C^{14} from radioactive C^{14}-carbon dioxide enters the amino-acids of leaves was determined. The pattern of labelling was very similar to that found for leaves of other species. The metabolic relationships existing between photosynthesis and amino-acid synthesis therefore do not seem to be affected by the large quantities of γ-MGA and γ-MG present in the leaves. γ-MGA and γ-MG only gained traces of radioactivity.

Experiments designed to study the incorporation of C^{14} from uniformly labelled C^{14}-alanine into the amino-acids of roots, immature leaves and cotyledons of seedlings and young plants indicated that the main site of synthesis of γ-MGA was the cotyledons.

Various C^{14}-labelled substrates were fed to germinating seeds and, after a period of growth, the specific activities of the amino-acids of seedlings receiving different treatments were determined. Comparison of the specific activities enabled certain deductions to be made concerning the probable biosynthetic pathways leading to γ-MGA and γ-MG. The results were consistent with the intact incorporation of pyruvate molecules, or another related 3-carbon-atom containing compound, into γ-MGA.

INTRODUCTION

IN recent years the application of the paper partition chromatographic method to the study of the nitrogenous constituents and metabolism of plants has revealed that a number of species contain unusual amino-acids that are not normally present in all plants; nor are they constituents of the proteins of the plants in which they occur. The presence of one or more such acids in a particular plant presumably indicates that the species is endowed with a complement of enzymes concerned with nitrogen metabolism that are additional to those usually encountered in plants having only a normal complement of amino-acids.

Earlier studies have shown that the groundnut plant (*Arachis hypogaea*) is an example of a species containing unusual amino-acids. In addition to a complement of those amino-acids normally present in proteins, these plants

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also contain substantial quantities of two unsaturated amino-acids, γ-methyleneglutamic acid (γ-MGA) and γ-methyleneglutamine (γ-MG). These acids each contain one ethylenic bond which links the single branch carbon atom to the γ-carbon atom of the main carbon chain (Done and Fowden, 1952). They are apparently absent from the dormant seed, but are produced during the early stages of seed germination and seedling growth, and rapidly become predominant over other free amino-acids. Their formation appears to be connected with the onset of the respiratory degradation of seed reserve materials, and it has been suggested, by analogy with known chemical conversions, that the substances may arise by condensation of two molecules of pyruvic acid, or other related three-carbon-atom substances, produced during this degradation (Fowden, 1954).

If this biosynthetic pathway was operative, γ-MGA would be expected to have a nitrogen-free, six-carbon-atom compound as its precursor. This role may be filled by γ-methylene-α-ketoglutaric acid, the keto acid analogue of γ-MGA, which has been shown to be a constituent of groundnut plants (Fowden and Webb, 1955). The keto acid, like γ-MGA, was produced in the very early stages of seedling growth (Webb and Fowden, 1955). γ-MGA might arise *in vivo* from the keto acid by the action of a suitable transaminase, widely distributed in different organs of the groundnut plant (Fowden and Done, 1953). The outline biosynthetic pathway is then possibly as in Fig. 1 below.

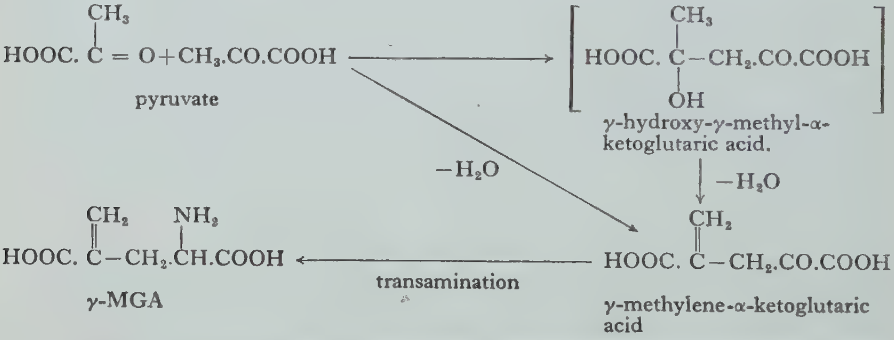
HOOC.C(CH₃)=O+CH₃.CO.COOH
pyruvate
HOOC.C(CH₃)(OH)–CH₂.CO.COOH
γ-hydroxy-γ-methyl-α-ketoglutaric acid.
−H₂O
−H₂O
HOOC.C(=CH₂)–CH₂.CO.COOH
γ-methylene-α-ketoglutaric acid
transamination
HOOC.C(=CH₂)–CH₂.CH(NH₂).COOH
γ-MGA

FIG. 1 shows in outline the possible conversions involved in the biosynthesis of γ-MGA.

γ-hydroxy-γ-methyl-α-ketoglutaric acid, a possible intermediate in this biosynthetic scheme, and its corresponding amino-acid, γ-hydroxy-γ-methylglutamic acid, have not been detected in groundnut plants, but have been reported as constituents of *Phyllitis scolopendrium* (Virtanen and Berg, 1955). It is possible that they have only transitory existences in groundnut plants and so never accumulate to detectable concentrations.

Other evidence presented in this earlier publication (Fowden, 1954) indicated that the amide (γ-MG) may be important as the compound in which the major quantities of nitrogen are translocated from the sites of nitrogen storage in the cotyledons to the sites of new growth and protein synthesis in the root and shoot apices.

The present paper reports experiments in which radioactive C^{14}-labelled substances have been widely used to obtain further information on the nitrogen metabolism of groundnut plants. The following questions have been the subject of particular study:

(*a*) Does the groundnut plant, which has γ-MGA and γ-MG present in large amounts, have a radically different overall scheme of nitrogen metabolism from that possessed by plants having normal amino-acid constitutions? Or does it merely have certain additional metabolic processes superimposed on the normal pattern? These questions have now been investigated in a general way, at least in so far as leaves are concerned, by determining whether the interrelationships existing between the processes of photosynthesis and amino-acid formation in groundnut plants are the same as those encountered in other species. Excised groundnut leaves were allowed to photosynthetically assimilate $C^{14}O_2$ for short periods of time and the subsequent incorporation of radioactivity into amino-acids was followed. The sequence in which different acids became labelled was compared with the sequence of amino-acid labelling occurring when leaves of soybean, a legume with a normal amino-acid complement, fixed $C^{14}O_2$ (determined by Racusen and Aronoff, 1954).

(*b*) Which organs of the groundnut plant are the most active sites of synthesis of γ-MGA and γ-MG? Previous work (Fowden, 1954) had indicated that the synthesis of these compounds probably took place predominantly in the cotyledons. In the present investigation C^{14}-labelled substrates ($C^{14}O_2$ or uniformly labelled C^{14}-alanine) have been fed to immature and mature leaves, and to root and cotyledon slices, in an attempt to confirm this finding. Comparisons of the rates of incorporation of radioactivity into the γ-MGA and the γ-MG present in the different tissues were made; these rates act as a measure of the amount of synthesis occurring in the various tissues.

(*c*) What is the pathway of γ-MGA and γ-MG synthesis? Various C^{14}-labelled substrates (uniform–C^{14}-glucose, 1–C^{14}-acetate (carboxyl labelled), 2–C^{14}-acetate (methyl labelled), 1–C^{14}-pyruvate (carboxyl labelled) and $KHC^{14}O_3$) have been used to test whether pyruvate, or a related 3-carbon-atom containing compound, is incorporated directly into the molecules, as suggested in the scheme of Fig. 1. The substances were fed to germinating seeds, and the extents to which various amino-acids became labelled with C^{14} were determined. A comparison of their relative activities after different substrate treatments enabled a definite conclusion to be reached concerning pyruvate incorporation.

(*d*) Can γ-MGA or γ-MG of high activity be formed in useful amounts by any of the above treatments? If this could be done, the amino-acids could be isolated, and used in experiments designed to determine whether they are readily degraded by groundnut plants, and, if so, by what biochemical mechanisms. A *useful* synthesis of C^{14}-labelled γ-MGA or γ-MG would be one in which an appreciable percentage of the C^{14} fed entered the compounds;

synthesis from $C^{14}O_2$ would be preferable, since this is the cheapest available source of C^{14}.

GENERAL METHODS AND MATERIALS

In this section only techniques that are common to most experiments will be described. Detailed treatments used in particular experiments will be described later when the results of the experiments are recorded.

All experiments involved the assimilation of various C^{14}-substrates by different organs of the groundnut plant, and, after an appropriate interval of time, the distribution of radioactivity in the amino-acids of the tissue was examined. The procedures involved in the assay of radioactivity in individual amino-acids were as follows:

Extraction of the tissues. The tissue was killed by dropping into hot 75 per cent. (v/v) ethanol solution (20 ml. per g. fresh weight of tissue). The tissue was then broken up with a pestle and mortar, and the extraction allowed to continue at laboratory temperature for 24 hours. The debris was then separated by centrifugation and re-extracted with a second portion of hot 75 per cent. ethanol. The two extracts were combined, whilst the plant debris was discarded. The combined extract was then evaporated to dryness in a current of warm air. The residue was dissolved in distilled water, any insoluble matter being removed by centrifugation. These solutions contained the free amino-acids of the tissue, together with other substances soluble in aqueous ethanol, e.g. sugars, sugar and triose phosphates, organic acids, &c.

Separation of the cationic fraction. Since the sugars, the phosphate esters, and the organic acids also became labelled with C^{14} in the experiments, it was found advisable first to separate the amino-acid fraction from these other radioactive substances in the extracts by the use of a cationic exchange resin as follows: Zeocarb 215 (60–80 mesh), previously activated with $2N \cdot HCl$, was packed as a slurry in distilled water into small columns (80 mm. × 6 mm. diam.). After washing the columns free of chloride, an appropriate volume of each extract, equivalent to about 2 g. fresh weight of the tissue used, was allowed to run through the columns at a rate of about 2 ml. per minute. Amino-acids and any other cations were absorbed by the resin, whilst anions and uncharged substances (phosphate esters, organic acids, sugars) were not. The latter substances were completely washed through the columns using a further 100 ml. of distilled water. The amino-acids were then displaced from the columns by 30 ml. of $3N \cdot NH_3$ solution, the eluates being collected. These were evaporated to dryness in a current of air and finally redissolved in distilled water, such that 1 ml. of the final solution was equivalent to 2 g. fresh weight of the tissue originally extracted.

PAPER CHROMATOGRAPHIC SEPARATION OF INDIVIDUAL AMINO-ACIDS

The chromatographic procedures were adjusted to obtain good separations of the acidic amino-acids and their amides on small sheets of Whatman No. 3 MM filter paper. 50 μl. of the final extracts ($\equiv$ 0·1 g. fresh weight tissue) were used for each chromatogram. With the solvent system, water-saturated phenol in the presence of NH_3, followed by butanol-acetic acid (Partridge, 1948), it was found advisable to develop the chromatogram for only 12 hours in phenol, when the solvent front had travelled 25 cm. down the paper. Development in the second solvent was then allowed to proceed for 48 hours. Whilst the leucines, phenylalanine and valine had often been carried off the end of the paper, proline, alanine, serine, glycine, threonine, arginine, and aspartic, glutamic, and γ-methyleneglutamic acids and their corresponding amides were well separated from each other on chromatograms that finally measured about 25×20 cm. The developed chromatograms were then used in one of three ways: (*a*) for the preparation of radioautographs, (*b*) for the assay of radioactivity in each amino-acid, or (*c*) for the quantitative determination of amount of each amino-acid.

(*a*) *Preparation of radioautographs.* Radioautographs were prepared from chromatograms by placing the latter in contact with 25×20 cm. sheets of Kodirex X-ray film (Code 7) for suitable periods of time, which depended upon the total radioactivity present in the cationic fraction applied to each chromatogram. After development of the radioautographs, the chromatograms were sprayed with 0·1 per cent. ninhydrin in ethanol; the positions of the amino-acids were revealed as coloured spots after gentle heating at 40–45°C. The radioautographs were then superimposed upon the sprayed chromatograms in order to determine which amino-acids had become marked with C^{14}.

(*b*) *Determination of the radioactivity in individual amino-acids.* The areas containing radioactive amino-acids were cut out from the chromatograms, and the amino-acids extracted from the pieces of paper by shaking vigorously with 10 ml. of hot distilled water. The radioactivity was then determined in measured fractions, after concentration, by the 'infinitely thin' sample method of Calvin, Heidelberger, Reid, Tolbert, and Yankwich (1949). The sample was pipetted on to recessed aluminium planchettes (2·1 cm. diam.). The drop was covered with a circle of lens tissue paper and the sample dried under an infra-red lamp at 70°C. It was then counted in a windowless gas flow (methane) proportional chamber connected to a scaler. As the samples possessed appreciable mass, correction was made for the self absorption of the radiation by the lens tissue and sample itself. The background count was determined after the assay of each sample.

Nelson and Krotkov (1955) have shown than when organically bound C^{14} is assayed in this way, the specific activity derived for the carbon does not greatly differ from the value obtained after conversion of the carbon to $BaCO_3$.

(*c*) *Quantitative determination of amino-acids.* These determinations were made only in the experiments where specific activities of amino-acids are reported. The method used has been described previously (Fowden, 1951, 1954).

Groundnut seedlings and plants. Seedlings were grown in vermiculite at 30°. Root and cotyledon slices were obtained from 3-day-old seedlings having radicles 4 cm. long. When leaves were required, plants were grown in a greenhouse, minimum temperature 30°, for either 1 or 4 weeks.

C^{14}-labelled substances were obtained from the Radiochemical Centre, Amersham. 1–C^{14}-*dl*-alanine was prepared from 1–C^{14}-pyruvate by catalytic hydrogenation of its 2:4-dinitrophenylhydrazone derivative (as Fowden and Webb, 1955).

RESULTS

Assimilation of $C^{14}O_2$ by leaves. Leaves which had just reached the fully expanded state were excised from 4-week-old plants by cutting at the bases of the petioles. During all further treatments until the leaves were killed, the bases of the petioles were kept immersed in distilled water. Batches of 4 leaves (about 2 g. total fresh weight) were placed in a glass vessel which was filled with CO_2-free air and sealed; the vessel was placed in an illuminated chamber at about 20° where the leaves received about 1,000 foot-candles of incident light. $C^{14}O_2$ was then liberated within the vessel by adding dilute hydrochloric acid to C^{14}-labelled barium carbonate (100 μc.; 1·1 mg.). Photosynthetic $C^{14}O_2$ fixation was allowed to proceed for $7\frac{1}{2}$, 15, 30, or 60 minutes, when the air was flushed from the vessel through N·NaOH solution to absorb remaining $C^{14}O_2$. The leaves were rapidly removed from the vessel and then were (*a*) immediately killed by dropping into 20 ml. of boiling 75 per cent. ethanol or (*b*) kept in the light or (*c*) kept in complete darkness for further periods of time up to 24 hours before being killed. The further treatments of each leaf sample were then as described in the methods section.

All the $C^{14}O_2$ supplied (0·12 ml.) was taken up by the leaves during the 30- and 60-minute periods of illumination. Approximately 25 per cent. of the $C^{14}O_2$ remained unabsorbed at the end of the $7\frac{1}{2}$-minute period, and 10 per cent. remained at 15 minutes. A large percentage of the $C^{14}O_2$ absorbed by the leaves remained either in this form or as bicarbonate when the leaves were analysed immediately after a $7\frac{1}{2}$-minute assimilation period. The assimilation of C^{14} into organic compounds was, however, substantially complete if leaves treated in this way were allowed to remain for a further 30 minutes, in light or dark, before analysis. Progressively smaller fractions of the absorbed $C^{14}O_2$ remained in inorganic form at the ends of the 15-, 30-, and 60-minute illumination periods respectively.

The cationic fractions, consisting mainly of amino-acids, rapidly became labelled with C^{14}. This was indicated by the fact that the activities of these fractions, expressed as percentages of the total radioactivity in the 75 per cent. ethanol extracts, were greater for the shorter $C^{14}O_2$ supply periods, e.g. 34 per cent. at 15 minutes, 20 per cent. at 30 minutes, and 5·4 per cent. at 60 minutes. The cationic fractions of leaves, which were kept in the light for further periods of time (30 minutes to 24 hours) after the supply of $C^{14}O_2$ was ended, contained at all times a fairly constant percentage of the total radioactivity in the soluble compounds (values ranged from 4·0 to 5·3 per cent., see Table I). This percentage was always higher when leaves were maintained in the dark. It was approximately constant for periods of 3 to 24 hours of dark storage at values ranging from 7·4 to 9·4 per cent., but leaves stored in the dark for shorter periods contained appreciably higher percentages of the total activity in the cationic fractions (see Table I).

TABLE I

Shows the radioactivity in the cationic fractions (amino-acids) of groundnut leaves, which were supplied $C^{14}O_2$ for short periods of time, and then maintained for further periods in light or darkness.

(The activities of the cationic fractions are expressed as percentages of the total radioactivity in all compounds soluble in 75 per cent. ethanol)

	Leaves maintained in light		Leaves maintained in dark	
Period of $C^{14}O_2$ supply (minutes)	Further time (hours)	Activity of cationic fraction (%)	Further time (hours)	Activity of cationic fraction (%)
7½	0·5	4·2	0·5	34·4
15	1	4·1	1	24·2
30	3	5·3	3	8·5
60	6	4·2	6	8·8
60	12	4·0	12	7·4
60	24	4·7	24	9·4

The pathway of incorporation of C^{14} into amino-acids of leaves was studied by determining the radioactivity of individual acids as a function of time. After the paper chromatographic separation of the amino-acids present in the cationic fraction of each leaf extract, all acids containing appreciable radioactivity were assayed. The radioactivity in a particular amino-acid was then calculated as a percentage of the total radioactivity determined for all acids. Generally the total activity of the amino-acids determined was equivalent to 65–80 per cent. of the radioactivity of the cationic fraction applied to the paper chromatogram. The incompleteness of the recovery of radioactivity is attributable mainly to losses arising during the chromatographic separation of the amino-acids, although some discrepancy also arises from the fact that substances of weak radioactivity present in the cationic fractions were not assayed.

The activities of the amino-acids present in leaves receiving different $C^{14}O_2$ treatments are recorded in Tables II, III, and IV.

It will be seen that the C^{14}-label appeared predominantly first in alanine and serine (15-minute period of Table II). The C^{14}-labelled alanine rapidly disappeared again (a marked fall was observed in the absolute counts per minute present in alanine as well as in its percentage contribution to the total activity of the amino-acids), whilst the C^{14}-labelled serine disappeared somewhat more slowly, especially in leaves stored in darkness (Table IV). After an hour glutamic acid had become predominantly marked. The level of C^{14}-labelling of glutamic acid, unlike that of alanine and serine, did not rapidly decline. In fact where leaves were stored in the dark after the original supply

TABLE II

Shows the incorporation of C^{14} into the amino-acids of groundnut leaves allowed to assimilate $C^{14}O_2$ in the light for short periods.

(Radioactivities in individual amino-acids are expressed as percentages of the total activity present in all amino-acids assayed)

	Radioactivities of individual amino-acids		
	% activities after 15 min. $C^{14}O_2$	% activities after 30 min. $C^{14}O_2$	% activities after 60 min. $C^{14}O_2$
Alanine	37·4	24·5	3·7
Serine	54·9	54·0	14·1
Glutamic acid	1·2	4·7	48·2
Aspartic acid	—	—	7·3
Asparagine	1·0	2·2	12·5
Glycine	5·5	7·1	3·4
γ-MG	—	—	2·9
Proline	—	—	0·9
Glutamine	—	—	1·5
Others	—	7·5	5·5

TABLE III

Shows the incorporation of C^{14} into the amino-acids of groundnut leaves maintained in the light for various periods of time after a short period of $C^{14}O_2$ supply.

(Radioactivities in individual amino-acids are expressed as percentages of the total activity present in all amino-acids assayed)

	% activities after following treatments					
Period of $C^{14}O_2$ supply (min.)	7·5	15	30	60	60	60
Light assimilation period (hours)	0·5	1	3	6	12	24
Alanine	28·7	4·8	1·1	2·0	—	—
Serine	44·3	9·0	2·0	2·3	1·7	1·0
Glutamic acid	15·4	72·1	76·7	50·8	22·6	15·8
Aspartic acid	0·1	1·3	0·9	0·8	—	0·7
Asparagine	6·2	6·1	6·1	6·9	9·2	13·5
Glycine	1·9	2·9	0·9	0·6	1·4	0·9
γ-MG	0·8	0·4	2·7	—	0·8	1·1
γ-MGA	—	—	1·0	—	—	—
Proline	—	—	3·7	23·8	52·1	56·9
Glutamine	2·6	3·4	2·7	2·0	3·8	3·0
Others	—	—	2·2	10·8	9·4	7·1

of $C^{14}O_2$ (Table IV), a nearly constant high percentage activity was found in glutamic acid at all times up to 24 hours. Storage of leaves in the light resulted in a slow disappearance, between the 3rd and 24th hours, of the C^{14}-labelled glutamic acid formed during the first hour. An even more marked contrast between the behaviours of leaves stored in the light and dark is seen in the C^{14}-labelling of proline. During the later periods of leaf storage in the light, when the C^{14}-marking of glutamic acid was decreasing, proline slowly

TABLE IV

Shows the incorporation of C^{14} into the amino-acids of groundnut leaves maintained in the dark for various periods of time after a short period of $C^{14}O_2$ supply.

(Radioactivities in individual amino-acids are expressed as percentages of the total activity present in all amino-acids assayed)

		% activities after following treatments				
Period of $C^{14}O_2$ supply	(min.)	15	30	60	60	60
Dark assimilation period	(hours)	1	3	6	12	24
Alanine		4·7	0·4	1·1	1·1	—
Serine		46·8	16·0	12·0	3·2	—
Glutamic acid		33·3	62·9	48·2	49·8	47·8
Aspartic acid		7·8	5·2	11·1	12·2	17·9
Asparagine		2·9	4·8	16·0	20·5	21·9
Glycine		3·2	2·1	—	3·9	2·2
γ-MG		—	0·7	0·3	—	1·5
γ-MGA		—	—	0·1	—	—
Proline		—	0·8	1·6	1·5	1·4
Glutamine		1·3	2·3	2·7	4·3	2·0
Others		—	4·8	6·9	3·5	5·3

gained a predominant C^{14}-label. This increase in the radioactivity of proline was accompanied by a marked rise in the concentration of free proline in the leaf. No significant C^{14}-labelling of proline occurred during the dark storage, and the amount of proline in the leaves remained low. Aspartic acid and especially asparagine slowly became appreciably labelled, more particularly in the dark.

A prominent feature of these experiments was the fact that although γ-MG and γ-MGA together make up the major proportion of the free amino-acid pool of the leaves, neither amino-acid became labelled with C^{14} other than in trace amounts. After certain of the treatments, one or more radioactive areas, additional to those corresponding to known amino-acids, were seen on the radioautographs and presumably resulted from the presence of other unknown cations in the leaf extracts. The radioactivity in these unknowns is listed in Tables II, III, and IV as 'Others'. The activity in any one unknown never represented more than 5 per cent. of the total activity of the cationic fraction.

The site of synthesis of γ-MGA and γ-MG. Three organs of the groundnut plant (immature leaves from 1-week-old plants, and the roots and cotyledons from 3-day-old seedlings) were tested for an ability to synthesize γ-MGA and/or γ-MG from C^{14}-labelled precursors. Uniform C^{14}-labelled *l*-alanine was chosen as a suitable substrate for several reasons. It rapidly penetrated into the cells of the different tissues. It also had the advantage, shown in the earlier $C^{14}O_2$ leaf fixation experiments, of being able quickly to enter the general metabolism of the plant, whilst its structure suggests that, like pyruvate, it may be a possible precursor of γ-MGA and γ-MG.

C^{14}-labelled alanine was introduced into leaflets of immature leaves using the technique of vacuum infiltration. The leaflets (fresh weight 0·2 g.) were immersed in 4 ml. of alanine solution (12·5 μc.; 0·17 mg.) and a partial

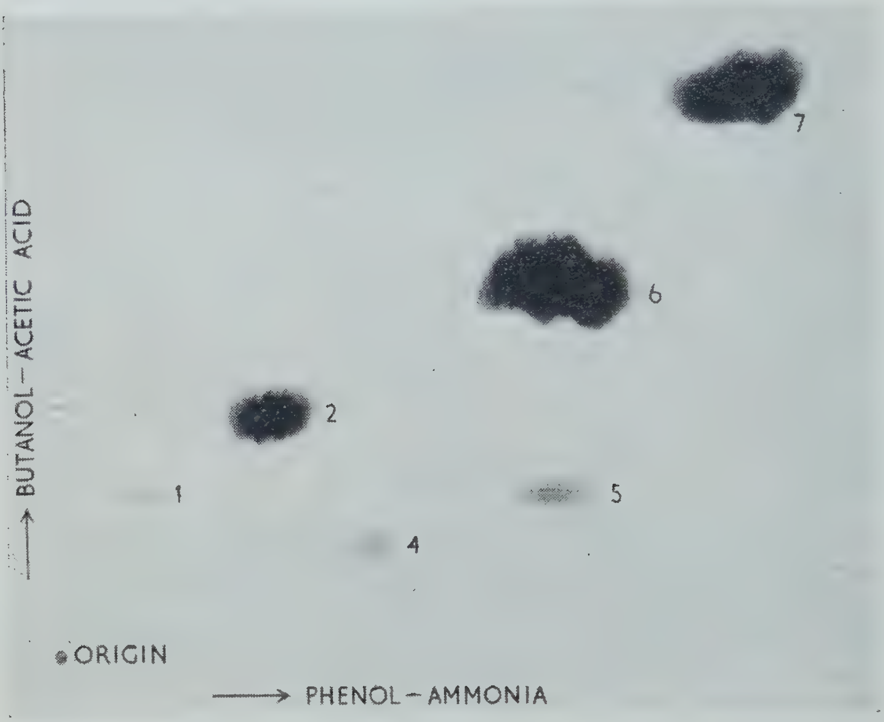

FIG. 2

FIGS. 2, 3 and 4 show reproductions of the radioautographs prepared from chromatograms of the cationic fractions of various groundnut tissues (0·1 g. fresh wt.) which had been supplied with C^{14}-alanine (see text). Fig. 2: cotyledon slices, incubation time 3 hrs. Fig. 3: root slices, incubation time 3 hrs. Fig. 4: immature leaves, incubation time 5 hrs. in dark.
Key to radioactive substances shown: 1, Aspartic acid; 2, Glutamic acid; 3, γ-MGA; 4, Asparagine; 5, Glutamine; 6, Alanine; 7, γ-aminobutyric acid; 8, Valine; X, non-ninhydrin reactive substance.

vacuum was applied several times. The leaflets were washed to remove adhering alanine solution, and, after blotting, were found to have increased in weight by about 25 per cent. of their original fresh weight. They were then kept in the dark under humid conditions for 5 hours at 30°C before being killed and extracted with 75 per cent. ethanol. A second set of leaflets was kept in the light after alanine infiltration.

When root and cotyledon tissue was used, slices of about 0·5 mm. thickness were cut, and these were shaken in a solution of C^{14}-alanine at 30° C. 0·60 g. of cotyledon slices were suspended in 5 ml. of solution (12·5 μc.; 0·17 mg. alanine), and 0·43 g. of root slices in a further 5 ml. (12·5 μc.; 0·17 mg.). At the end of 3 hours the slices were rinsed free of alanine, and then killed and extracted with 75 per cent. ethanol.

Portions of the cationic fractions obtained from each tissue extract were then chromatographed for the preparation of radioautographs and the assay of radioactivities in each amino-acid as previously described.

The pattern of C^{14}-labelling produced in the amino-acids of the three tissues is most easily seen by reference to reproductions of the radioautographs (Figs. 2, 3, and 4). These indicate that the C^{14}-labelled alanine supplied was rapidly metabolized in all tissues, and the distribution of the C^{14}-label between other amino-acids was widespread. In leaves maintained in the dark (Fig. 2), glutamic acid became most heavily marked, whilst aspartic acid, γ-aminobutyric acid, and valine gained appreciable radioactivity. Trace amounts of C^{14} also entered proline and asparagine. The labelling pattern of leaves kept in the light was very similar. In root slices (Fig. 3), the extent of C^{14}-labelling was found to decrease in the series γ-aminobutyric acid, glutamic acid, glutamine, asparagine, and aspartic acid respectively. Radioactivity was found in γ-MGA only in the experiment using cotyledon slices (Fig. 4). Glutamic acid and γ-aminobutyric acid also became appreciably marked, whilst serine, glutamine, aspartic acid, and valine were weakly labelled with C^{14}. γ-MG was not detectably labelled. The radioactivities of these amino-acids present in 0·1 g. fresh weight of treated cotyledon tissue, expressed as counts per minute, were as follows: residual

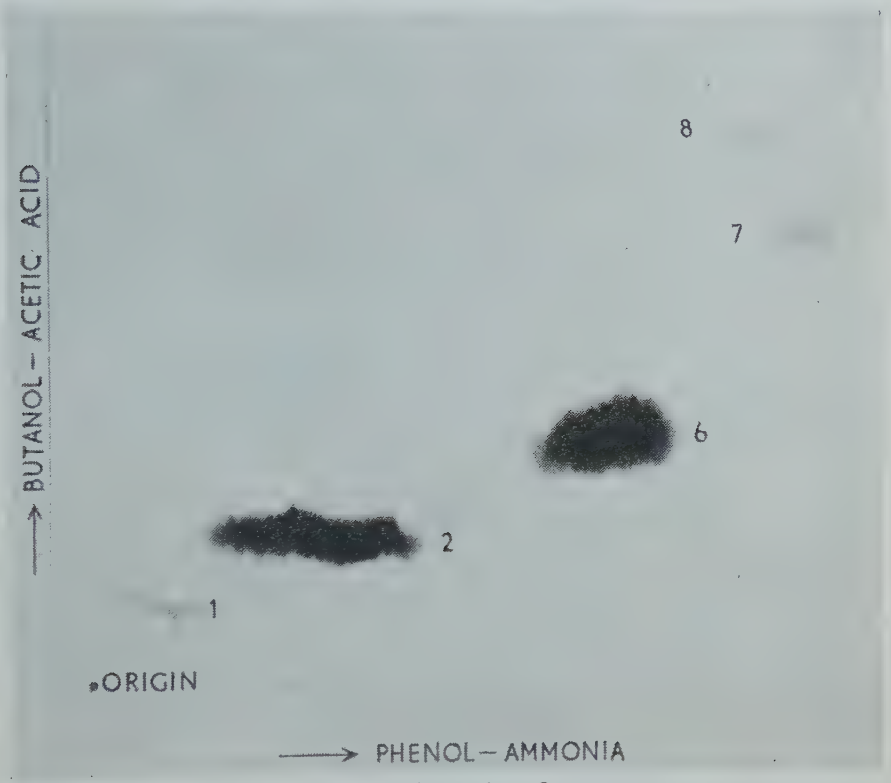

FIG. 3. For Legend see fig. 2.

alanine 15,630, glutamic acid 960, γ-MGA 713, γ-aminobutyric acid 506, valine 208, glutamine 181, serine 169, and aspartic acid 144. An additional radioactive substance was seen on the radioautograph (X on Fig. 4), but it did not coincide with any detectable ninhydrin reacting spot on the original chromatogram. It may be of some significance to note that the position of this substance on the radioautograph corresponded very closely to that known to be occupied by γ-hydroxy-γ-methylglutamic acid, although no further evidence was obtained regarding the chemical nature of unknown X. γ-MGA therefore became nearly as strongly labelled with C^{14} as did glutamic acid: this result is in sharp contrast with that obtained using leaves or root slices, where no detectable activity was found in γ-MGA, although glutamic acid became appreciably labelled in both tissues.

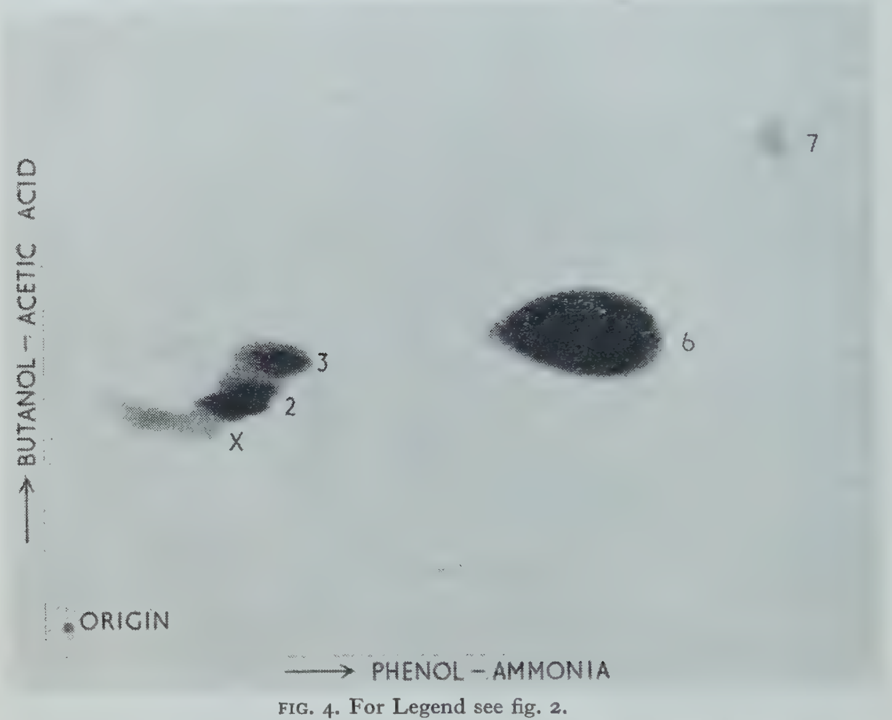

FIG. 4. For Legend see fig. 2.

Precursors in the biosynthesis of γ-MGA and γ-MG. The main aim of these experiments was to determine whether the three-carbon-atom chain of pyruvate or some related molecule was incorporated as an intact unit into γ-MGA and γ-MG, or whether the γ-methylene compounds were formed by condensation of a two-carbon-atom unit. Since it was already known that γ-MGA and γ-MG are formed rapidly in the first 3 days of seedling growth (Fowden, 1954), it seemed appropriate that this period of active synthesis should be used to study the utilization, by the germinating seeds, of various C^{14}-labelled substances in the synthesis of the amino-acids.

The following C^{14}-labelled compounds were used: uniform C^{14}-glucose

(1·3 mg.), 1–C^{14}-sodium acetate (1·9 mg.), 2–C^{14}-sodium acetate (1·6 mg.), 1–C^{14}-sodium pyruvate (1·2 mg.), and $KHC^{14}O_3$ (0·55 mg.). Each substance contained 100 μc. of radioactivity. Five similar groups of 6 groundnut seeds were selected and each group was allowed to imbibe 0·2 ml. of water containing one of the C^{14}-marked substances at 30° C. The solutions were imbibed completely by the germinating seeds in 18 hours. They were then transferred and allowed to grow for a further 48 hours on moistened filter-paper pads in sterilized Petri-dishes at 30°, after which they were killed and extracted with 75 per cent. ethanol. Chromatograms prepared from the cationic fraction of each extract were used to prepare radioautographs, and to assay the amounts and the radioactivity of individual amino-acids as described previously.

The amount of radioactivity present in the combined free amino-acids of the seedlings after the three-day period varied considerably for the different treatments. When 2–C^{14}-acetate was the substrate, about 4 per cent. of the supplied activity was ultimately found in the amino-acids. The corresponding percentages for the other C^{14}-substrates were: 1–C^{14}-acetate, 1·1 per cent.; uniform C^{14}-glucose, 1·2 per cent.; 1–C^{14}-pyruvate, 0·2 per cent.; $KHC^{14}O_3$, 0·3 per cent.

The activities of the individual amino-acids are given for each treatment in Table V, where they are expressed as counts per minute per μmol. of amino-acids, i.e. as specific activities.

TABLE V

Shows the radioactivities of individual free amino-acids present in groundnut seedlings grown at 30° C. for 3 days after the supply of different C^{14}-labelled substrates in the water of imbibition.

(Activities are expressed as counts per minute per μmol. of amino-acid)

C^{14}-compound supplied	Activities of amino-acid							
	Proline	γ-MG	γ-MGA	Glutamic acid	Glutamine	Aspartic acid	Asparagine	Alanine
Uniform C^{14} glucose .	1,167	5,470	6,410	2,160	1,750	2,780	2,160	6,860
1–C^{14}-sodium acetate .	2,897	1,950	2,500	1,600	1,570	982	3,210	960
2–C^{14}-sodium acetate .	11,120	7,800	10,400	6,460	3,880	4,320	6,850	2,320
1–C^{14}-sodium pyruvate	347	1,820	1,670	183	210	*	48	239
$KHC^{14}O_3$ . . .	212	1,062	1,030	382	350	2,506	2,297	1,064

* No assay of activity possible because a non-ninhydrin reactive, radioactive substance overlapped the aspartic acid spot on the chromatogram.

In order to be more useful when making comparisons between different treatments, the specific activity of each amino-acid relative to that of glutamic acid (≡ 1·00) for the same treatment has been calculated and is given in Table VI.

It will be seen that the relative extents to which amino-acids became labelled with C^{14} differed markedly. Whereas γ-MGA and γ-MG possessed the highest specific activities after 1–C^{14}-pyruvate feeding, asparagine and aspartic acid were most actively labelled when $KHC^{14}O_3$ was used. After the feeding of labelled acetate or glucose, the values of the relative specific activities of different amino-acids tended to be more nearly equal. The different patterns of amino-acid labelling resulting in these experiments are discussed later in relation to the pathways whereby γ-MGA and γ-MG are synthesized.

TABLE VI

Shows the radioactivities of individual free amino-acids present in groundnut seedlings grown at 30° C. for 3 days after the supply of different C^{14}-labelled substrates in the water of imbibition.

(Activities in counts per minute per μmol. of amino-acid are expressed relative to the activity of glutamic acid taken as 1·00)

	Relative specific activities of amino-acids							
C^{14}-compound supplied	Proline	γ-MG	γ-MGA	Glutamic acid	Glutamine	Aspartic acid	Asparagine	Alanine
Uniform C^{14}-glucose .	0·54	2·53	2·97	1·00	0·81	1·29	1·00	3·18
1–C^{14}-sodium acetate .	1·81	1·22	1·56	1·00	0·98	0·61	2·01	0·60
2–C^{14}-sodium acetate .	1·72	1·21	1·61	1·00	0·60	0·67	1·06	0·36
1–C^{14}-sodium pyruvate	1·89	9·95	9·13	1·00	1·15	*	0·26	1·31
KHC^{14}O$_3$ . .	0·55	2·78	2·69	1·00	0·92	6·56	6·01	2·79

* No value—see Table V.

The ability of cotyledon slices to synthesize γ-MGA from alanine (demonstrated in the previous section) was used in a further experiment designed to test whether the three-carbon unit was incorporated intact into γ-MGA. Cotyledon slices (0·2 g. samples) were gently shaken for 3 hours at 30° in 1 ml. of either uniform-C^{14}-*l*-alanine (15 μg.; 1·25 μc.) or 1–C^{14}-*dl*-alanine (30 μg.; 2·5 μc.). The cotyledon slices were then extracted in the normal way with 75 per cent. ethanol and the radioactivity present in γ-MGA, glutamic acid, alanine, serine, and aspartic acid was determined and compared for the two treatments. The results are given in Table VII. The activity in alanine is due to assimilated but unmetabolized substrate. γ-MGA became labelled with C^{14} in both treatments, but it is noteworthy that its

TABLE VII

Shows the radioactivity of various free amino-acids present in cotyledon slices shaken for 3 hours at 30° C. in either 1–C^{14}-*dl*-alanine (2×10^6 c.p.m.) or uniform-C^{14}-*l*-alanine (1×10^6 c.p.m.).

(Data are expressed as counts per minute (c.p.m.) in the amino-acids extracted from 0·1 g. of cotyledon tissue)

	Corrected c.p.m. determined for uniform-C^{14}-alanine treatment		Corrected c.p.m. determined for 1–C^{14}-alanine treatment	
Amino-acid	Absolute	Relative to glutamic acid	Absolute	Relative to glutamic acid
Glutamic acid . . .	1,050	1·00	52	1·00
γ-MGA	592	0·56	232	4·46
Alanine	23,103	—	18,790	—
Aspartic acid . . .	187	0·17	0	0·00
Serine	238	0·23	106	2·04

activity, when compared to that of glutamic acid, was far higher when 1–C^{14}-alanine was used than when uniform-C^{14}-alanine was the substrate. The same conclusion is true for the labelling of serine.

DISCUSSION

The results of the experiments described above permit an answer to be given to some of the original questions posed, whilst to others a partial answer becomes possible.

The $C^{14}O_2$-fixation experiments leave no doubt that the pathways of amino-acid synthesis from nitrogen-free precursors are generally the same in groundnut leaves as in those of other species previously examined. Some amino-acids are considered to arise from their corresponding keto acids either by direct amination, e.g. glutamic acid from α-ketoglutaric acid, or by transamination, e.g. alanine from pyruvic acid, aspartic acid from oxalacetic acid, and glutamic acid from α-ketoglutaric acid. When a leaf is allowed to fix $C^{14}O_2$ photosynthetically, the order in which these amino-acids become labelled with C^{14} will be dependent upon the sequence in which their corresponding keto acids become radioactive. The fact that in groundnut leaves the C^{14}-label first enters alanine and serine (Table II) and was only later found in glutamic acid and aspartic acid (and asparagine) agrees with the earlier findings of Racusen and Aronoff (1954) using soybean leaves. It also provides indirect evidence that groundnut leaves possess normal metabolic pathways by which pyruvate arises first from the primary fixation product, 3–phosphoglyceric acid, by glycolysis. This is subsequently converted to α-ketoglutaric acid, followed by oxalacetic acid, by the reactions of the tricarboxylic acid cycle.

The early appearance of considerable activity in serine is in agreement with the earlier observations of Calvin and Benson (1949) using *Scenedesmus* and *Chlorella*, and the results of later studies of Racusen and Aronoff (1954) with soybean leaves, and Towers and Mortimer (1956) with sugar-beet leaves. Whilst the present results do not allow one to infer with certainty the biosynthetic pathway of serine in groundnut leaves, the fact that serine reaches a peak activity before glycine (see Table II) indicates that it is not synthesized from the latter amino-acid, as was reported by Tolbert and Gailey (1955) for wheat leaves. Serine may in fact be the precursor of glycine (as found in animal tissues by Shemin, 1946, and by Nye and Zabin, 1955). Uncertainty still surrounds the nature of the immediate nitrogen-free precursor of serine (see Nye and Zabin, 1955).

The later stages of C^{14}-incorporation into amino-acids were affected by the light conditions to which the groundnut leaves were subjected. The most marked difference between leaves stored in the light and dark was found in the behaviour of proline. This imino-acid accumulated to high concentrations during the later stages of light storage (6–24 hr.), whereas it could not be detected in leaves stored in the dark even after 24 hours. Since the proline accumulating also became appreciably marked with C^{14}, its production must be due, at least in part, to *de novo* synthesis. Whilst some might arise also by protein hydrolysis, this seems less likely because storage in the dark, which would tend to increase protein hydrolysis, did not result in proline accumulation. Studies with micro-organisms (Vogel and Davis, 1952; Vogel, 1953; Fincham, 1953; Vogel and Bonner, 1954) have indicated that proline is synthesized from glutamic acid via glutamic-γ-semialdehyde and an unsaturated heterocyclic compound, Δ^1-pyrroline-5-carboxylic acid, i.e. by a pathway involving two reductive stages. If the manner of proline synthesis is the same in higher plants, the effect of light on the synthesis of the imino-acid in groundnut leaves might be mediated through its indirect stimulation of reduced coenzyme production.

The amino-acids represent a variable percentage of the total radioactivity of the aqueous ethanol soluble compounds of the leaves. It will be seen from Tables I–IV that this percentage was highest when alanine and serine, alone of the amino-acids, were actively labelled. As the labelling became more general in the amino-acids, their percentage contribution to the total activity of the soluble compounds decreased. Table I also shows that this percentage was higher for leaves maintained in the dark after $C^{14}O_2$ feeding than in similar leaves kept in the light. Presumably in the light, a higher proportion of the C^{14} is converted to sugars and related compounds, and less proceeds along the catabolic pathways that lead from phosphoglyceric acid to amino-acids.

Only trace amounts of radioactivity were found in γ-MGA and γ-MG of leaves after $C^{14}O_2$ feeding (see Tables II–IV). Since the C^{14}-label became widely distributed in other compounds during the experimental period, the conclusion must be drawn that the unsaturated amino-acids were synthesized only very slowly by leaves, and that the main site of their synthesis in the young plants must be located elsewhere. This conclusion was further confirmed by the experiments in which uniformly labelled C^{14}-alanine was fed to immature leaves. Although synthetic processes might be expected to be more active in leaves that were in a state of rapid growth, there was again no formation of γ-MGA or γ-MG in 5 hours. Similarly, no synthesis was detectable in root slices in 3 hours. In contrast, when C^{14}-alanine was fed to cotyledon slices over a 3-hour period, the γ-MGA present in the tissue had become labelled with C^{14} to a greater extent than all other amino-acids except glutamic acid. There can then be little doubt that the major site of synthesis of γ-MGA and γ-MG is in the cotyledons. This work with C^{14}-labelled substrates therefore complements earlier work (Fowden, 1954), in which γ-MGA and γ-MG were shown to arise during the early stages of seedling growth.

In the experiments designed to study the biogenesis of γ-MGA and γ-MG, the C^{14}-labelled substrates fed to the seedlings (uniform-C^{14}-glucose, 1–C^{14}-acetate, 2–C^{14}-acetate, 1–C^{14}-pyruvate, and $KHC^{14}O_3$) were chosen particularly to test the hypothesis that pyruvate or a derived three-carbon-atom molecule was incorporated intact into the unsaturated amino-acids. Assuming that the reaction sequences of the tricarboxylic acid (TCA) cycle are operative in groundnut seedlings (unpublished experiments indicate that several of the cycle acids are oxidized by particles prepared from seedlings), it is possible to make predictions concerning the relative amounts of C^{14} that will be introduced into the γ-MGA, γ-MG, glutamic, and aspartic acids and their amides present in the seedlings.

If pyruvate or a derived three-carbon compound is introduced intact into γ-MGA and γ-MG, their specific activities, relative to those of other amino-acids, after seedlings had been fed 1–C^{14}-pyruvate should be markedly different to the ratios obtained after any one of the other four C^{14}-labelled substances was supplied. 1–C^{14}-pyruvate would give relatively high activities in γ-MGA and γ-MG compared with the activity of glutamic or aspartic acids if the above hypothesis is correct, since the C^{14} atom would directly enter γ-MGA and γ-MG. Only slight labelling of glutamic and aspartic acids and their amides would be expected, for pyruvate molecules undergoing decarboxylation to give acetyl-coenzyme A would liberate their αC^{14} atom as $C^{14}O_2$, and the remaining β and γ carbon atom moiety of pyruvate, now as acetyl-coenzyme A, entering the TCA cycle would be unlabelled (see Fig. 5). Labelled aspartic acid could only be formed from any oxalacetate molecules produced directly from labelled pyruvate by carboxylation (Wood-Werkman reaction); in this case the oxalacetate would be labelled either in the δ position only if a molecule of inactive CO_2 was fixed or in the δ and η positions if a molecule of liberated $C^{14}O_2$ was re-utilized (Fig. 5). Alternatively, oxalacetate labelled just in the η position would be produced by fixation of $C^{14}O_2$ into unlabelled pyruvate. Some labelled glutamic acid could be produced from α-ketoglutarate arising from oxalacetate with an ηC^{14}-label. The single δ or η label of oxalacetate could also become equally distributed between the δ and η positions of the molecule if it were first converted to fumarate by a reversal of part of the TCA cycle, and the symmetrical molecule of fumarate were then reconverted to oxalacetate. In this way labelled glutamic acid could also be produced from oxalacetate originally possessing only a δC^{14} atom.

When uniform C^{14}-glucose was used, four of its six C^{14} atoms would enter the TCA cycle, via acetyl coenzyme A, and so produce labelled glutamic and aspartic acids. Similarly, after esterification with coenzyme A, the C^{14} atoms of either 1– or 2–C^{14}-acetate would enter the cycle and subsequently these amino-acids. Labelled pyruvate would also be produced from each of these substrates, so γ-MGA and γ-MG could become radioactive. Since these substances lead to a more direct labelling of glutamic and aspartic acids and their amides, they would now be expected to gain higher absolute activities than when 1–C^{14}-pyruvate was used; their specific activities relative to those of γ-MGA and γ-MG should also be higher when glucose or acetate was used as a source of C^{14}-label.

These predictions are essentially borne out by the determinations of relative specific activities recorded in Table VI. The experimental data therefore give general support to the original hypothesis.

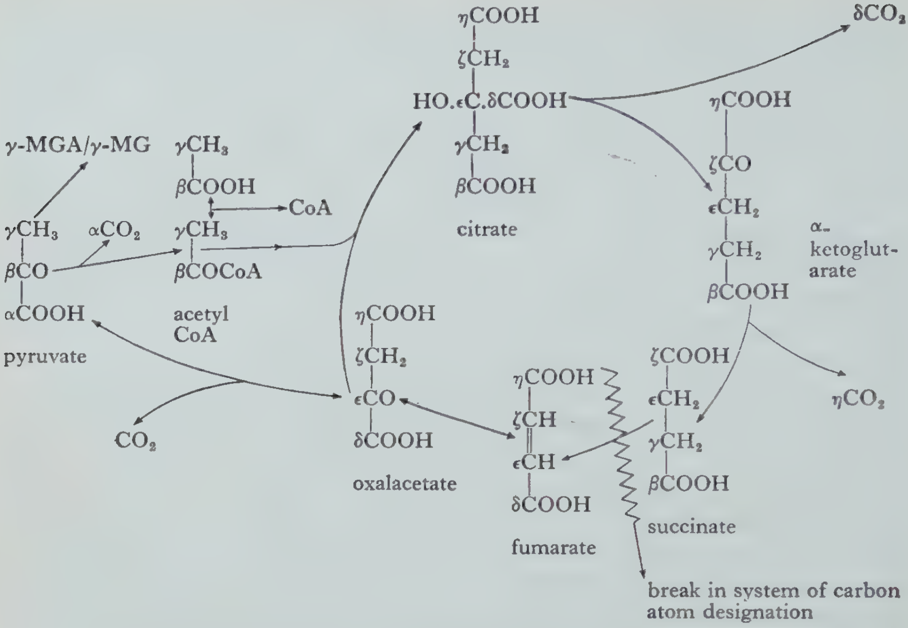

FIG. 5 shows the probable metabolic pathways of the carbon atoms of pyruvate and oxalacetate.

If the carbon chain of pyruvate underwent fission before its moieties entered γ-MGA and γ-MG, then the entry of C^{14} into the two amino-acids would again be by indirect pathways (compare the production of labelled glutamic and aspartic acids from 1–C^{14}-pyruvate above), involving the re-utilization of the $\alpha C^{14}O_2$ produced by decarboxylation. The position would then be similar to the experiment in which C^{14} was supplied as $KHC^{14}O_3$, when labelling of amino-acids is dependent upon reactions involving direct fixation of $C^{14}O_2$. Under these conditions the relative specific activities of aspartic acid and asparagine would be expected to be high, since the Wood-Werkman type of reaction is probably the most important fixation mechanism. The results obtained using $KHC^{14}O_3$ (see Table VI) are in agreement with these predictions, the specific activities of aspartic acid and asparagine being higher than those of γ-MGA, γ-MG, glutamic acid, and glutamine. The labelling pattern produced by 1–C^{14}-pyruvate is seen to be in sharp contrast; γ-MGA and γ-MG have specific activities that are far greater than those of other amino-acids. The labelling of γ-MGA and γ-MG therefore cannot be explained by assuming a preliminary fission of the pyruvate, followed by a re-utilization of the $C^{14}O_2$ produced.

The experiments in which two forms of labelled alanine (uniform-C^{14} and 1–C^{14}) were supplied to cotyledon slices are also in agreement with an incorporation of the intact three-carbon unit. Whilst C^{14} from both forms of alanine entered γ-MGA, glutamic and aspartic acids were marked far more heavily when uniformly labelled alanine was used. The behaviour of serine, which is probably also formed from alanine without fission of the three-carbon atom chain, is similar to that of γ-MGA, the activity present in each of these two amino-acids after 1–C^{14}-alanine treatment being about half that in the same acid after uniform-C^{14}-alanine was supplied.

Table V indicates that the specific activities of γ-MGA and γ-MG are higher than those of most of the other amino-acids irrespective of the C^{14}-substrate used. This is probably largely explained by the fact that all the other amino-acids recorded in Table V are constituents of the seed proteins whereas γ-MGA and γ-MG are not (Done and Fowden, 1952). Molecules of glutamic and aspartic acids, asparagine, glutamine, proline, and alanine synthesized from the C^{14}-labelled substrate are therefore diluted by other inactive molecules arising during the protein hydrolysis accompanying germination, and their specific activities are correspondingly reduced; in contrast no dilution of newly formed γ-MGA and γ-MG can occur in this way.

Table V also shows that the specific activities measured for γ-MGA and γ-MG are very similar irrespective of the C^{14}-substrate used. This finding supports the logical concept that γ-MGA and γ-MG arise by the same biosynthetic pathway; by comparison with the known route of glutamine synthesis in plants (Elliot, 1953), γ-MG might be expected to arise from γ-MGA and NH_3 by a reaction requiring adenosine triphosphate. If one makes similar comparisons of the specific activities of glutamine and glutamic acid, and of asparagine and aspartic acid, the values for the amide and acidic amino-acid do not always correspond so closely. The differences found may again be the result of protein hydrolysis, which probably produced unequal amounts of amide and corresponding acidic amino-acid, and so causes unequal dilution of the newly synthesized, radioactive molecules.

The proportion of the total C^{14} supplied to the germinating seedlings that was present in the amino-acid fraction upon their analysis at the third day of growth varied for the different C^{14}-substances used. 2–C^{14}-acetate was utilized for amino-acid synthesis to a greater extent than any of the other substances tested. This can be explained by the fact that the carbon skeletons of most amino-acids are derived directly or indirectly from intermediates of the TCA cycle. The C^{14} atom of 2–C^{14}-acetate remains in intermediary metabolic processes for a longer period than the C^{14} atoms of the other substances; no C^{14} is liberated as $C^{14}O_2$ from either type of acetate until the molecule has entered the TCA cycle, whereas uniform-C^{14}-glucose loses one-third, and 1–C^{14}-pyruvate loses all its C^{14} before their residues enter the TCA cycle via acetyl-CoA. Having entered the TCA cycle, C^{14} is liberated more slowly from 2–C^{14}-acetate than from 1–C^{14}-acetate because, in the former case, respiratory $C^{14}O_2$ is not produced by the reactions of the TCA cycle until the C^{14} atoms commence their third revolution. $C^{14}O_2$ is produced from 1–C^{14}-acetate during its second turn of the cycle.

The low percentage incorporation of C^{14} from 1–C^{14}-pyruvate into amino-acids indicates that the decarboxylation pathway to acetyl-coenzyme A is probably far more important in the seedlings than the alternative pathway of carboxylation of pyruvate to oxalacetate.

The main conclusion, reached from the experiment data of Tables V and VI, is that at least one molecule of pyruvate enters γ-MGA and γ-MG intact without prior fission of the three-carbon chain. The mechanism involved may be a direct condensation of two molecules of pyruvate (see earlier scheme). This could best be proved if sufficient activity could be introduced into γ-MGA or γ-MG from 1–C^{14}-pyruvate to enable stepwise degradation and the determination of the activity of each of the six carbon atoms to be undertaken. The two carboxyl carbons would be expected to contain virtually all the activity if such a direct condensation occurred. The poor percentage incorporation of C^{14} from 1–C^{14}-pyruvate into amino-acids meant that the total activity present in γ-MGA or γ-MG was not sufficient for a degradation of this type to be carried out. However, the data of Tables V and VI cannot exclude the possibility that only one molecule of pyruvate enters γ-MGA intact, the other three carbon atoms having independent origins. It is also impossible to say whether pyruvate or a derived three-carbon compound are incorporated directly into γ-MGA or whether they are first converted to an intermediary four- or five-carbon atom compound.

The maximum incorporation of C^{14} into γ-MGA and γ-MG together was obtained when 2–C^{14}-acetate was used; in this case about 1·4 per cent. of the C^{14} supplied was found in the two substances. This low percentage conversion from a relatively expensive C^{14}-substance did not provide an attractive method for obtaining radioactive γ-MGA. No useful conversion of the cheaper $C^{14}O_2$ to γ-MGA or γ-MG could be demonstrated.

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The Morphology of Protonema and Bud Formation in the Bryales

BY

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With Plate and forty text-figures

ABSTRACT

The development of protonema from spore in aseptic culture was followed in species representing 14 genera and 7 orders of the Bryales. In no case could a distinction be made between sharply defined 'chloronema' and 'caulonema' stages.

In all species, with the exception of the anomalous *Cinclidotus fontinaloides*, the protonema developed a markedly heterotrichous habit, with prostrate filaments of relatively unlimited growth and erect filaments of more restricted development. The two types of filament were usually distinct in cellular characteristics as well as in the direction of growth. In some cultures a third type of filament grew downwards into the medium.

Shoot buds were found to occupy diverse positions in different species, but the commonest position was near the base of a primary filament of the erect branch system. Shoot buds were formed as readily in artificial light of sufficient intensity as in natural daylight.

INTRODUCTION

SINCE the first reasonably accurate description of the spore germination of a moss, by Hedwig (1782), there have been many investigations of the protonema of the Bryales, but our knowledge of this juvenile phase of the gametophyte is still far from complete. The early workers, e.g. Müller (1874), Sachs (1875), Goebel (1882), described the *primary protonema*, i.e. the protonema developed directly from the spore, as composed of two principal types of branched uniseriate filaments, green filaments rich in chlorophyll which extended over the surface of the substratum, and pale or brownish rhizoids which usually penetrated the latter. Müller considered that the two types of filament are morphologically homologous, and claimed that under appropriate conditions each kind can undergo transformation into the other. Correns (1899) accepted the essential homology of the two types of filament but proposed the convenient term *chloronema* for the green surface filaments. A similar distinction between rhizoids and chloronema was also recognized in the *secondary protonema*, which is usually taken to include the normal rhizoids of the leafy moss plant, and the rhizoids or chloronema produced by regeneration from portions of the gametophyte or even of the sporophyte.

Although it is now generally agreed that the rhizoids and chloronema are modifications of the same basic type of filament, it is usually conceded that they possess fairly well-marked characteristics when present in their typical

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forms. According to Correns, the commonest type of chloronema filament has abundant chlorophyll, hyaline cell-walls, and perpendicular cross-walls, while typical rhizoids have little or no chlorophyll, pigmented, usually brownish, cell-walls, and oblique cross-walls. Correns observed that all possible transitions can occur between the two extreme forms.

Subsequent workers have reported additional differences between the rhizoids and chloronema. Westerdijk (1907) pointed out that branches of successive order retain the same width in the chloronema, but in the rhizoids are progressively narrower. It has also been emphasized that the rhizoids are positively geotropic (e.g. by Němec, 1904; Bischoff, 1912; E. and O. Pringsheim, 1935; Heitz, 1942; Fitting, 1950). Bauer (1942) even considered that this tropic response is the most important characteristic of the rhizoids, since it is the feature most closely connected with their physiological function of absorption of nutrients.

There has been some controversy as to the factors responsible for the differentiation of protonema filaments into chloronema or rhizoids. For a considerable time, the view prevailed that light is decisive (Müller, 1874; Sachs, 1875), rhizoids being produced in darkness and chloronema in the light, but it has since been shown repeatedly that typical rhizoids may be formed abundantly in liquid or agar cultures exposed to a strong light intensity. Both Goebel (1930) and Westerdijk (1907) considered that the failure of the rhizoids of intact moss plants to produce chloronema on exposure to light is a correlation phenomenon, for as previously shown by Correns (1899) when rhizoids are detached from the shoot they can readily develop into typical chloronema.

Although rhizoids are almost invariably present at the base of the leafy moss plants, they are not always produced by the primary protonema. It would appear from the literature that different species behave differently in this respect. Only relatively few species produce a rhizoid directly from the spore (Zederbauer, 1902*a*; Schoene, 1906; Janzen, 1913; Ruhland, 1924; Lorch, 1931; Woesler, 1933) but such a method of spore germination has been described frequently for *Funaria hygrometrica* (e.g. by Müller, 1874; Schoene, 1906; Gurlitt, 1918; Patschovsky, 1928; Bauer, 1942; Heitz, 1942; Fitting, 1950) and species of *Physcomitrium* (e.g. by Goebel, 1889, Rabe, 1905; Schoene, 1906; Meyer, 1947; Kachroo, 1954). The formation of a rhizoid as a regular feature in the germination of the spore has also been reported for *Polytrichum commune* (Wigglesworth, 1947) and *Atrichum aundulatum* (Becquerel, 1906; Fitting, 1950).

Some workers have related the production of rhizoids on the primary protonema to the concentration of nutrients in the culture medium, and there is fairly general agreement that a reduction in the concentrations of mineral nutrients, particularly of nitrogen sources, promotes the formation and growth of rhizoidal branches (Schoene, 1906; Gurlitt, 1918; Patschovsky, 1928; Bauer, 1942; and Fitting, 1950). It has frequently been reported that these rhizoidal filaments of the primary protonema are less strongly differentiated than those of the leafy shoot. They penetrate downwards into the medium and are narrower and paler than the other protonemal filaments, but often lack the brown pigment and oblique cross-walls of typical shoot-rhizoids. A number of authors (e.g. Servettaz, 1913; Patschovsky, 1928; Woesler, 1933; Sironval, 1947, 1952, and Van Andel, 1952) have even questioned whether true rhizoids occur on the primary protonema and would restrict the term rhizoid to the filaments of this nature produced secondarily by the leafy shoot.

A new approach to the problem of the occurrence of rhizoids on the primary protonema was introduced fairly recently by Sironval (1947) in his investigations on *Funaria*. Sironval considered that previous workers had paid insufficient attention to the sequence of events in the development of the protonema. He claimed that two distinct protonemal stages could be recognized. The first stage was produced immediately on spore germination and consisted entirely of green filaments with rather scanty and irregular branching. It was proposed that the term *chloronema* should be restricted to this stage, which was said to persist for about 20 days before degenerating to leave only a few persistent apical cells as the starting-point of the next phase, the *caulonema* stage. This stage was so-called because it alone was believed to be capable of giving rise to the buds of leafy shoots. According to Sironval the principal caulonema filaments had certain rhizoidal characteristics, such as oblique cross-walls, pigmented cell-walls, and small chloroplasts, but bore branches with perpendicular cross-walls, hyaline cell-walls, and numerous well-developed chloroplasts. Sironval evidently believed that these caulonema filaments correspond to the protonema-rhizoids of previous workers and that the recognition of the caulonema as a necessary preliminary to the formation of leafy plants would explain many of the difficulties in the earlier accounts. But although it is clear that a number of workers have described the 'caulonema' filaments as protonema-rhizoids, it seems equally clear that the protonema-rhizoids are not always of 'caulonema' nature.

A number of subsequent workers have accepted Sironval's concept of caulonema. Bopp (1952) had shown independently that the buds of *Funaria* appear on branches of rhizoid-like filaments of the protonema which he termed *protonema-rhizoids*, but in later papers (1954, 1955) he adopted the term caulonema for his protonema-rhizoids. Van Andel (1952), however, in a detailed study of the protonema of *Funaria*, failed to observe any stage corresponding to the caulonema of Sironval.

It is quite evident that there are still considerable differences of opinion concerning some of the principal features of the morphology and development of the moss protonema. There can be little doubt that the conclusions of a number of authors have been based on the study of an insufficient range of species, frequently on *Funaria hygrometrica* alone, and are not generally valid. It also seems likely that in some investigations the conditions of culture have been unfavourable for normal development. The present investigation has been based on the study of the protonema of a range of representative genera of the Bryales, and particular attention has been directed towards the more controversial aspects of protonema development. A preliminary note of some of the principal findings has appeared previously (Allsopp and Mitra, 1956).

MATERIALS AND METHODS

Observations were made on aseptic cultures prepared from ripe spores of the following mosses, comprising species of 14 genera from 7 orders: *Atrichum undulatum* (Hedw.) P. Beauv., (Polytrichales); *Pleuridium acuminatum* Lindb., *Ceratodon purpureus* (Hedw.) Brid., *Dicranella heteromalla* (Hedw.) Schp., (Dicranales); *Tortula muralis* Hedw., *Tortula subulata* Hedw., *Cinclidotus fontinaloides* (Hedw.) P. Beauv., (Pottiales); *Grimmia pulvinata* (Hedw.) Sm., (Grimmiales); *Funaria hygrometrica* Hedw., (Funariales); *Leptobryum pyriforme* (Hedw.) Wils., *Pohlia nutans* (Hedw.) Lindb., *Bryum capillare* Hedw., *Mnium hornum* Hedw., (Eubryales); *Brachythecium rutabulum* (Hedw.) B. & S., and *Eurhynchium riparioides* (Hedw.) Jennings, (Hynobryales). In the following account, except where the specific name is necessary, the generic name alone is used to indicate the particular species in culture.

Although not essential the technique of aseptic culture offered many advantages and was used throughout the work. It has been previously employed by Becquerel (1906), Forest Heald (1898) and especially successfully by Servettaz (1913), v. Ubisch (1913), and most subsequent workers.

In the present investigation, ripe but unopened capsules, after removal of the calyptra, when present, were dipped first in absolute alcohol, to ensure ease of wetting, then in calcium hypochlorite solution for a minute or so. After washing in sterile distilled water, the capsules were opened by sterile needles and the spores scattered over nutrient agar in Petri-dishes. When germination was observed, usually after a few days, small groups of spores were transferred to the appropriate culture vessels.

The cultures were grown under various conditions. The basic medium was a half-strength Knop's solution with addition of Berthelot's mixture of trace elements, thus corresponding to that used by Gautheret (1942) and by one of us (Allsopp, 1952) in investigations on the water-fern, *Marsilea*. Cultures were prepared in glass tubes (12×2·5 cm.), Petri-dishes, and 100 ml. Erlenmeyer flasks. Some cultures were grown in a liquid medium, but normally agar was added at a concentration of 1 per cent. for culture tubes and Petri-dishes, and 0·5 per cent. for Erlenmeyer flasks. Other cultures were grown on filter paper dipping in the liquid medium. The pH of the medium was usually adjusted to 6·0, but in one series of cultures the initial pH was 4·0. Comparison of cultures at the two pH levels indicated that within the range employed pH has little effect.

In culture vessels containing solid media, a small group of germinating spores from the original sowing was placed centrally, leaving almost the whole surface of the substratum available for the growth of the protonema. This method was adopted in the belief that the random sowing of spores employed by some earlier investigators had possibly obscured some of the principal characteristics of protonema development. It had already been shown by E. and O. Pringsheim (1935) and Bopp (1952) that the principal filaments from a small inoculum spread radially over the medium giving rise to circular protonemal cultures. In the present work no attempt was made to work with single-spore cultures, since preliminary investigations confirmed the finding of Bopp that cultures prepared from small groups of spores showed essentially the same behaviour. We also found that small portions of old protonema cultures on transfer to new media would produce radial cultures similar to those developed directly from the spores. Parallel sets of cultures were subjected to three different conditions of lighting. One series was exposed to natural daylight in the northern window of the laboratory, the other two series were kept in a culture room provided with two units of fluorescent tubes ('Natural') one unit giving a light intensity of 100 foot-candles at the level of the cultures, the other a light of 500 foot-candles. The light was automatically switched on for 16 hours and off for 8 hours. In the laboratory the temperature was unregulated, but in the culture room was maintained at a fairly constant temperature of from 22–24° C.

MORPHOLOGICAL OBSERVATIONS

Spore germination

The conditions necessary for the germination of the spores have probably been studied more frequently than any other aspect of the development of the protonema. The accounts are very contradictory and it seems likely that some of the discrepancies may be referred to the use of spores of different age, different periods of storage, or from capsules developed under different environmental conditions. It is also probable that the behaviour of the spores is considerably affected by the nature of the culture medium. Much attention has been directed to the effects of light on germination (summarized by Meyer, 1948) and there can be little doubt that the germination of many species is retarded or even completely checked in darkness, at least on media containing only inorganic nutrients. This topic was not specially investigated in the present work, but in the one species tested in this way, *Dicranella heteromalla*, we failed to obtain germination on mineral agar in darkness, while rapid germination was obtained on the same medium in artificial light.

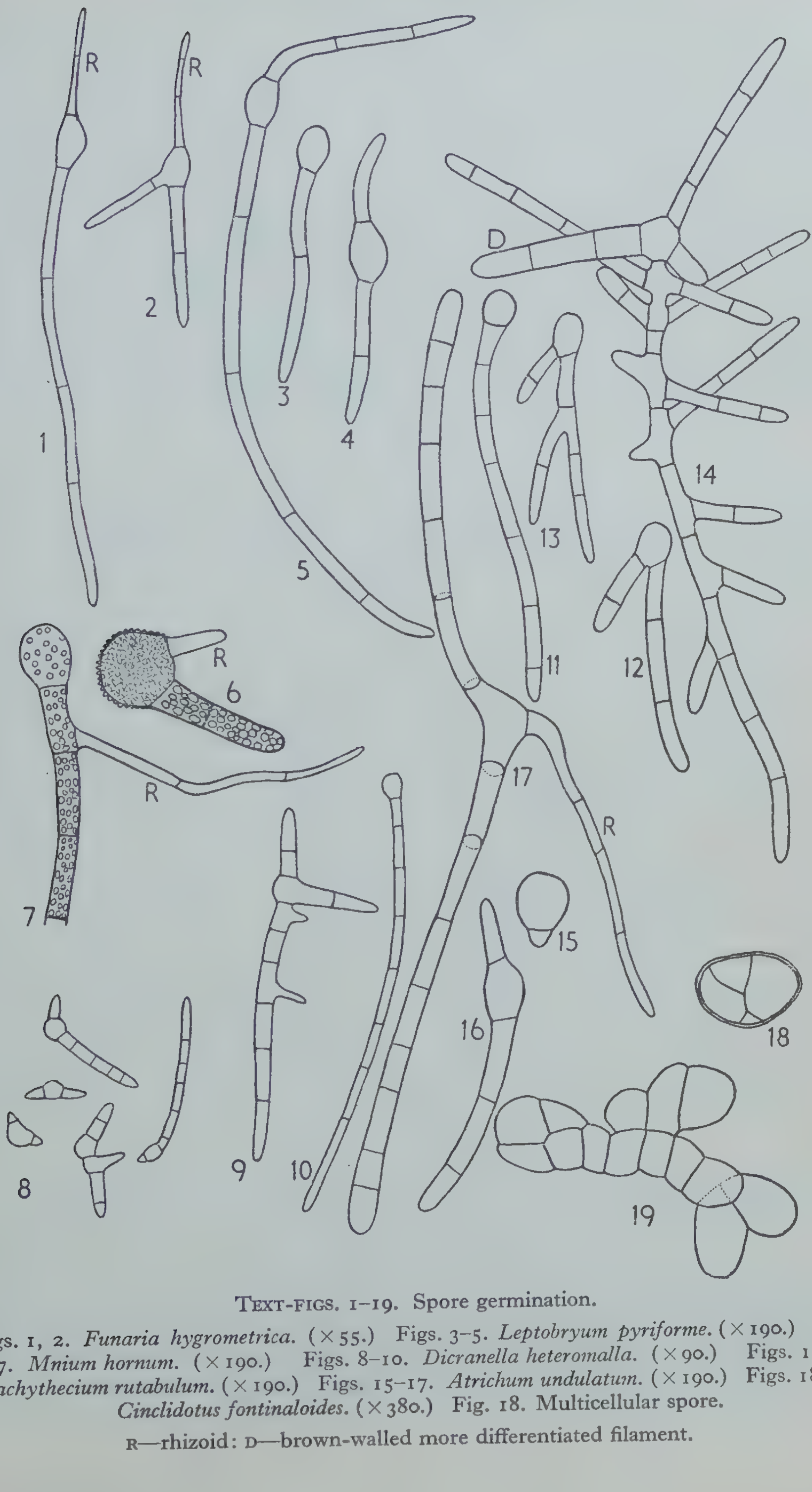
In the preparation of the protonemal cultures, we obtained consistently good germination by using spores from fully ripe, but still unopened capsules. Spores in this condition germinated usually within a few days or at the most within a fortnight. This uniformly early germination is in opposition to the findings of Servettaz (1913) who believed that in most species which produce their spores in spring, germination is immediate, e.g. in *Dicranella heteromalla*, *Grimmia pulvinata*, and *Funaria hygrometrica*, while in species producing their spores in late autumn or winter, e.g. *Polytrichum juniperinum*, *Atrichum undulatum*, and *Brachythecium rutabulum* there is a delay of from 2 to 6 months in germination. Servettaz found that spores of *Atrichum* *undulatum* sown on December failed to germinate until 10 March, but in the present investigation spores of *Atrichum undulatum* sown on 19 October germinated after 4–5 days, and spores of *Brachythecium rutabulum* germinated in less than a fortnight. These observations indicate that the two types of germination found by Servettaz are not of constant occurrence and cannot be explained by the season of ripening.

The first stages of spore germination were studied in seven of the genera grown in culture, namely in *Atrichum, Dicranella, Cinclidotus, Funaria, Leptobryum, Mnium,* and *Brachythecium.* Except in *Cinclidotus*, no unusual features were observed. In agreement with the account of previous workers (see Introduction) the formation of a narrower paler rhizoidal branch directly from the spore occurred frequently only in *Funaria* (Text-figs. 1, 2) and *Atrichum* (Text-figs. 15–17) but was observed occasionally in the cultures of *Mnium* (Text-figs. 6, 7) which had been exposed to the low intensity of artificial light. In *Funaria* the growth of this spore-rhizoid was soon arrested, while in *Mnium* and particularly in *Atrichum*, the rhizoidal branches attained considerable length, as previously observed by Wigglesworth (1947) in *Polytrichum.* In the other genera investigated (*Dicranella*, Text-figs. 8–10; *Leptobryum*, Text-figs. 3–5; *Brachythecium*, Text-figs. 11–14) the spores gave rise to one or more germ-tubes with the characteristics of chloronema, and no rhizoid was produced directly from the spore. Our observations on *Dicranella heteromalla* and *Brachythecium rutabulum* thus differ from those of Servettaz (1913) who described the production of small cellular masses in the germination of these species. It seems likely that this type of germination was determined by his cultural conditions, for in our own cultures such masses were never observed.

A special type of germination was found in *Cinclidotus fontinaloides*, in which we were able to confirm the observation of Elssmann (1923) that the ripe capsule contains both unicellular and multicellular spores. The latter had undergone one, two, or three divisions, giving rise to a small group of cells separated by thin walls and surrounded by a common exospore (Text-fig. 18). No greater number of divisions was seen in any spores still within the capsule, and *Cinclidotus*, which is perhaps the only British moss producing multicellular spores, thus provides an interesting intermediate step to those few mosses, such as *Dicnemon*, which normally form large multicellular spore-bodies within the capsule (Lorch, 1931). On the liberation of the spores, further divisions of both unicellular and multicellular types produced small more or less globular masses of cells from which several usually short-celled filaments developed (Text-fig. 19) although filaments with longer cells were occasionally formed. Elssmann figured similar early stages of germination but did not succeed in obtaining further development of the protonema.

Development and differentiation of the protonema: the heterotrichous habit

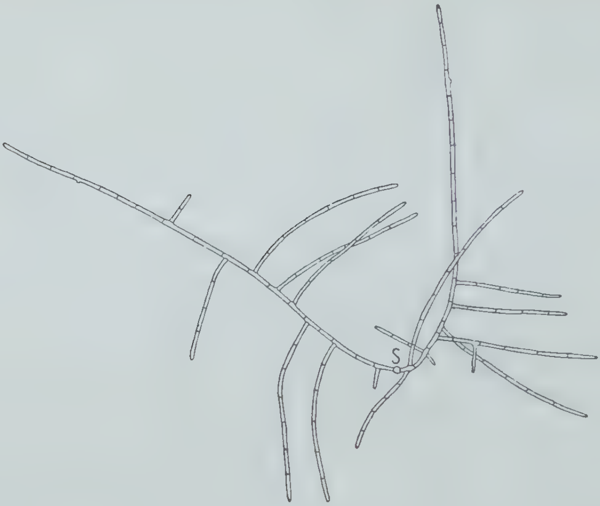
In all the genera under investigation, with the exception of *Cinclidotus*, which will be considered later, the general features of protonema development



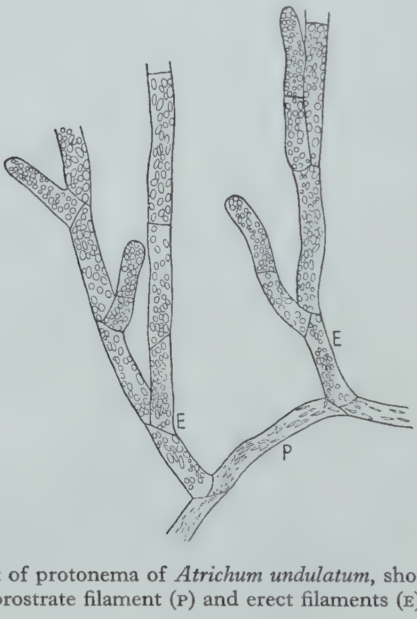
TEXT-FIGS. 1–19. Spore germination.

Figs. 1, 2. *Funaria hygrometrica.* (× 55.) Figs. 3–5. *Leptobryum pyriforme.* (× 190.) Figs. 6, 7. *Mnium hornum.* (× 190.) Figs. 8–10. *Dicranella heteromalla.* (× 90.) Figs. 11–14. *Brachythecium rutabulum.* (× 190.) Figs. 15–17. *Atrichum undulatum.* (× 190.) Figs. 18, 19. *Cinclidotus fontinaloides.* (× 380.) Fig. 18. Multicellular spore.

R—rhizoid: D—brown-walled more differentiated filament.



TEXT-FIG. 20. Young protonema of *Leptobryum pyriforme*, consisting only of prostrate system. S—spore-cell. (×45.)



TEXT-FIG. 21. Part of protonema of *Atrichum undulatum*, showing differentiation between prostrate filament (P) and erect filaments (E). (×190.)

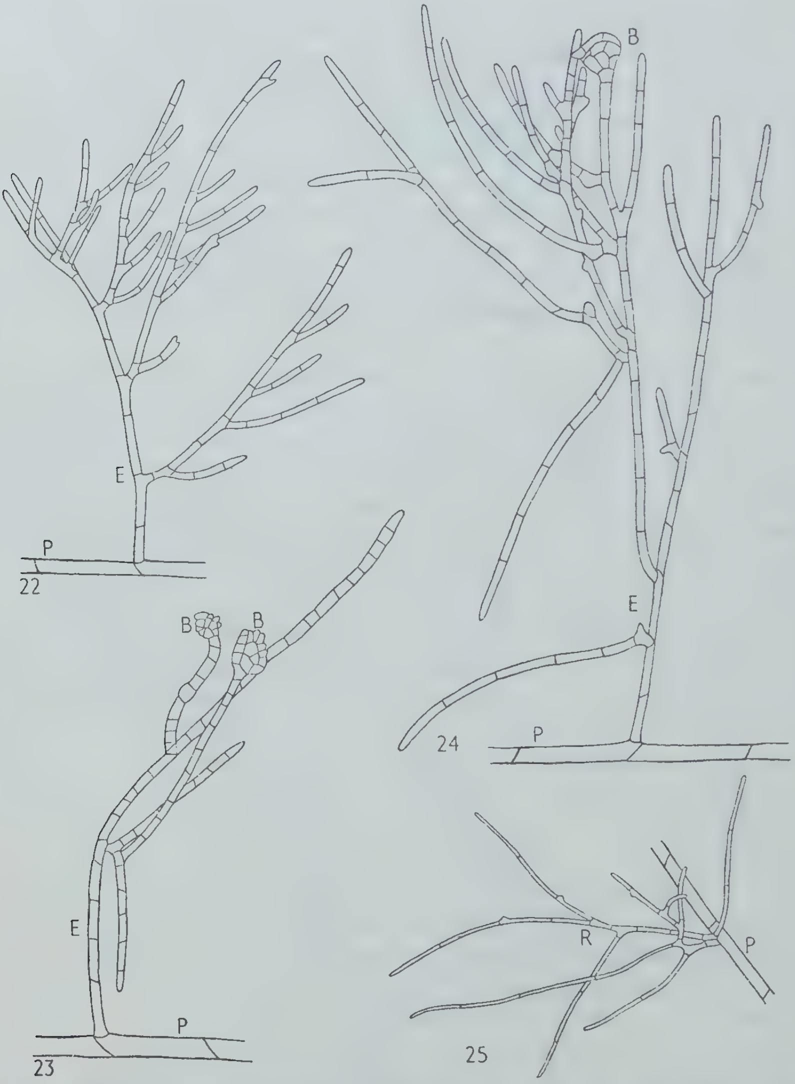
were essentially similar. The chloronema filaments produced from the spore spread more or less horizontally through or on the substratum, at first in all directions, but soon a markedly radial growth pattern was established, probably as a result of mutual interference between the principal filaments (Plate 1, Figs. 1, 2, 3).

For the first two or three weeks after germination, there was little if any differentiation of the protonema (Text-fig. 20), and except for a few pale rhizoidal branches in *Atrichum* and *Mnium*, all the filaments retained the characteristics of typical chloronema, i.e. abundant chloroplasts, hyaline cell walls, and perpendicular cross-walls. In this early period of protonema development the branching was more irregular than in later stages. In a well-developed protonema, only one branch is formed per cell and arises as a lateral protrusion immediately behind the acroscopic cross-wall, but in the young protonemata the branches originated from any part of the cell, and frequently more than one branch was produced by a single cell (Text-fig. 14). Both Sironval (1947) and Van Andel (1952) observed a similar irregularity of the branching in the young protonema of *Funaria*. The features of the young protonemata as described above were found in all the genera under consideration and evidently correspond to what Sironval referred to as the 'chloronema' stage.

As development of the protonema continued an increase in the differentiation of the originally uniform chloronema was gradually established. This progressive and continuous development of the protonema was in marked contrast to the sharp separation of 'chloronema' and 'caulonema' stages described by Sironval (1947). His description of the degeneration of the former and the persistence of only a few apical cells as the starting-point of the caulonema phase, strongly suggests that unfavourable cultural conditions had led to the death of most of the filaments of his cultures, but that a few more resistant apical cells persisted and became adapted to the existing conditions. This interpretation is supported by the results of Van Andel (1952) who, in agreement with our own observations, found in *Funaria* that the growth of the protonema was continuous, without any degeneration of a primary chloronema as a preliminary to the formation of a distinct caulonema stage.

In all the genera studied in the present work, with the exception of *Cinclidotus*, a distinction soon became apparent between filaments of relatively unlimited growth and lateral filaments of more restricted development. The principal filaments, some of which had originated as lateral branches, constituted a prostrate system creeping on or through the substratum, while most of the lateral filaments formed an aerial system arising vertically or obliquely from the prostrate system (Plate 1, Fig. 4; Text-figs. 21–24). The pattern of growth is thus markedly *heterotrichous*, to adopt the term employed by Fritsch (1935) for the corresponding condition in the algae.

The heterotrichous habit is particularly well seen in *Funaria*, where the erect branches are fairly well separated and of comparatively restricted development. In the fully differentiated protonema they occur as small, branched, bright green tufts providing a striking contrast with the strong, dark-brown filaments of the prostrate system. The latter filaments are prominent in Petri-dish cultures, where they are clearly visible to the naked eye as fine dark lines running towards the periphery of the medium (Plate 1, Fig. 1). In many cultures the filaments follow a strikingly spiral course. Similar spiral patterns were described by E. and O. Pringsheim (1935) for some of their protonema cultures, and had previously been observed in cultures of a bacterium (Pringsheim, 1918). In the present work the phenomenon was observed in cultures of *Funaria*, *Pohlia*, and *Leptobryum*, in which the spiral was consistently anticlockwise in the majority of cultures, although occasional clockwise cultures were also seen. After various experiments, Pringsheim (1918) arrived at the view that the production of the spiral pattern was not



Text-figs. 22–25. Parts of protonemata of *Grimmia pulvinata* (Fig. 22); *Bryum capillare* (Fig. 23); and *Tortula muralis* (Fig. 24), showing prostrate filament (P) and erect system (E); B—bud. Fig. 25. *Pohlia nutans*, prostrate filament (P) with rhizoids (R). All figures. (×60.)

directly due to external agencies, but was inherent in the growth of the bacterium itself. It seems likely that the growth pattern of the protonema cultures is also a genetical characteristic.

The extent of development of the aerial and prostrate systems, although influenced to some extent by the cultural conditions, particularly by the light intensity, is a characteristic of the individual species, so that some of the cultures are readily identified by their general appearance. In *Mnium*, as in *Funaria*, the filaments of the erect system are low and fairly widely spaced, while in *Dicranella* and *Grimmia* (Text-fig. 22) the filaments of this system are also low but closely crowded. In the majority of the genera investigated (*Atrichum*, Text-fig. 21; *Ceratodon*, Allsopp and Mitra (1956) Fig. 1; *Pohlia*, *Leptobryum*; *Bryum* and *Eurhynchium*) the prostrate system is still quite extensive, but the aerial system is considerably better developed than in *Funaria*. The erect branches arise close together in this group of genera and frequently become intertwined to form conical tufts, which rise vertically above the medium in the uniform lighting of the culture room, but are directed towards the window in the daylight cultures (Plate 1, Fig. 9). In older cultures of this type the individual tufts often become obscured by the formation of a dense turf of the aerial filaments. In *Pleuridium* and *Tortula*, the prostrate system spreads slowly, but the aerial system is correspondingly well developed (Text-fig. 24; Allsopp and Mitra (1956) Figs. 1 and 2).

Apart from the striking difference in the direction of growth, the filaments of the erect and prostrate systems show considerable differences in cellular features, such as diameter. Usually, as in *Funaria*, *Bryum*, and *Tortula* (Text-figs. 23, 24) the prostrate filaments are stouter than the erect, but in *Atrichum* (Text-fig. 21) the reverse is true. In all the species investigated, the filaments of the erect system are composed of richly chlorophyllous cells with the characteristics of typical chloronema, although in *Pohlia* and *Atrichum* the principal filaments of this system have frequently oblique cross-walls, while in *Ceratodon*, *Pleuridium*, *Leptobryum*, and *Bryum*, such walls occur occasionally. It is a well-marked characteristic of the aerial system that the cells are not easily wetted, while those of the prostrate system present no difficulty in this respect.

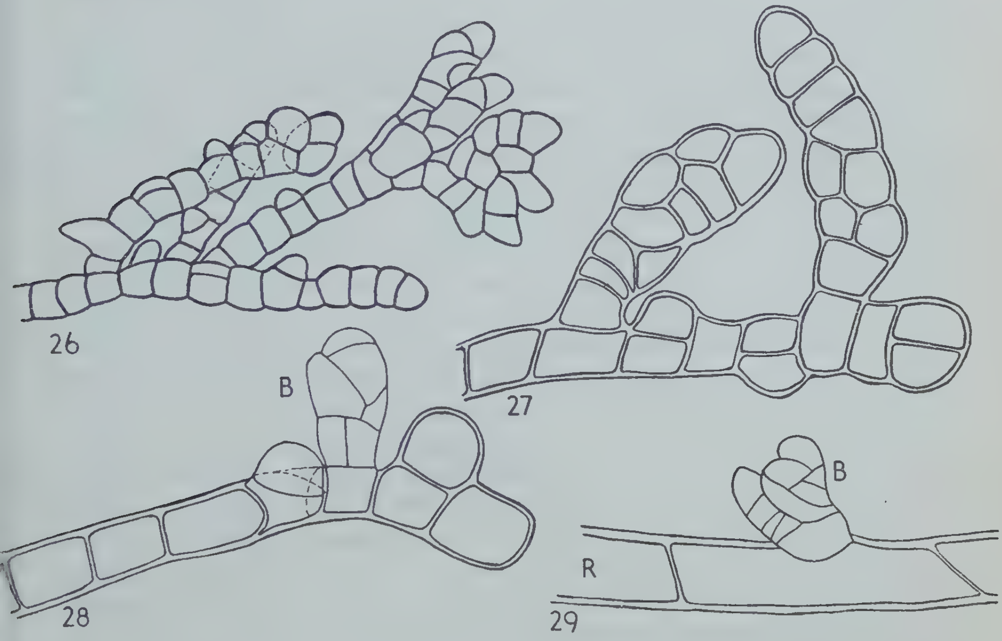
In strong contrast to the aerial filaments, those of the prostrate system have usually brown cell-walls, oblique cross-walls, and are relatively deficient in chloroplasts. Of the species investigated, *Ceratodon*, *Funaria*, *Leptobryum*, *Mnium*, *Bryum*, and *Eurhynchium* had cell-walls deep brown in colour, while in other species (*Tortula*, *Dicranella*, *Pleuridium*, and *Atrichum*) they were much paler. The pigmentation is influenced by cultural conditions for the walls were always darker in cultures grown on media containing glucose.

As previously observed by Servettaz (1913) and Woesler (1933) the chloroplasts are usually very elongated in the older cells of prostrate filaments of the protonema (Text-fig. 21). Woesler also noted that the elongation of the chloroplasts in successive cells corresponds fairly closely with the similar elongation of the nucleus. Changes in the shape of the nucleus were not followed in the present investigation, but Woesler's observations have recently been confirmed by Bopp (1955) in a study of the development of the cell and nucleus in the 'caulonema' of *Funaria*. Bopp also observed that the browning of the cell walls is associated with the changes in nuclear form.

The prostrate system of the fully differentiated protonema, with the characteristics described above, evidently corresponds to the 'caulonema' of Sironval (1947) and to the 'protonema-rhizoids' of Bopp (1952). Some of the features of the prostrate system are commonly found in typical rhizoids, and Sironval believed that the formation of the 'caulonema' as a necessary stage in the complete development of the protonema would account for previous reports of the occurrence of rhizoids on the primary protonema. There can indeed be little doubt that many workers have described the filaments of the prostrate system as rhizoids, but it should be emphasized that these filaments have little in common with true rhizoids, from which they differ, apart from their plagiotropic growth, in their production of richly chlorophyllous branches of the aerial system; furthermore the apical cell contains numerous chloroplasts, while that of typical rhizoids is almost or entirely colourless. It should also be mentioned that the characteristics of the typical prostrate systems develop gradually and a single filament may possess chloronema characteristics near its apex and the features of 'protonema-rhizoids' in the fully differentiated cells further removed from the apex. Filaments with the cellular characteristics of fully differentiated prostrate filaments were not usually found on the young protonema, but in *Brachythecium* (Text-fig. 14) well-differentiated prostrate filaments were sometimes produced almost directly from the spore.

The principal plagiotropic filaments of the prostrate system cannot be described as true rhizoids, but certain of their branches, which run downwards into the medium and produce branches of the same type, rather than typical chloronema filaments, would be correctly placed in this category. These protonema-rhizoids occur in most cultures and are usually deficient in chloroplasts and narrower than the typical chloronema filaments (Text-fig. 25; Allsopp and Mitra (1956), Fig. 2). Under our cultural conditions at least, they are rarely as differentiated as the rhizoids of the leafy shoot, and lack the brown pigment and oblique septa of these typical rhizoids.

The tropic responses of the moss protonema still require a thorough study. Both Sachs (1879) and Czapek (1898) considered that the protonema of *Funaria* is diaphototropic, while Czapek also claimed that the protonema is diageotropic. It seems likely that both authors studied the response of the prominent prostrate system and ignored the relatively inconspicuous aerial system. The filaments of the aerial system are in fact strongly positively phototropic and incline markedly towards one-sided light (Plate 1, Fig. 9). In their studies of the tropisms of moss protonema E. and O. Pringsheim (1935), although marking no reference to the differentiation of aerial and prostrate filaments, did notice that lateral branches originated on the light side of the principal filaments. Czapek (1898) had previously claimed that in *Funaria* all the lateral branches arise in a plane at right-angles to the direction of the light, and that this is a case of *photoauxesis* i.e. an influence of the light direction on the origin and development of lateral organs. The true rhizoids of the protonema are positively geotropic (p. 96). and negatively phototropic (E. and O. Pringsheim, 1935; Heitz, 1942; Fitting, 1950. See also Plate 1, Fig. 9).



TEXTS-FIGS. 26–29. *Cinclidotus fontinaloides.*

Figs. 26, 27. Parts of protonema. Figs. 28, 29. Bud formation, on protonema filament (Fig. 28) and on rhizoid (Fig. 29).

B—bud. R—rhizoid. Fig. 26. (× 190.) Figs. 27–29. (× 380.)

Cinclidotus. In *Cinclidotus* the pattern of growth was considerably different. Instead of forming a felt on the surface of the medium, the protonema developed as compact, dark-green, more or less globular masses (Plate 1, Fig. 5). On detailed examination it was found that the apparently solid tissue was formed from short-celled irregularly branched filaments which frequently showed longitudinal division of occasional cells (Text-figs. 26, 27). Apart from the early stages following spore germination, the protonema of *Cinclidotus* does not appear to have been seen by previous workers, and there may be doubt whether the growth pattern observed will always be found. It was obtained at the three different light intensities, on media both with and without glucose, and since young leafy shoots with strong rhizoids were also produced, similar effects possibly exist under natural conditions.

The range of observations makes it likely that the heterotrichous habit of the protonema will be characteristic of the Bryales as a whole. It is perhaps rather surprising that this regularity in morphological construction has received little attention from previous workers, but the true nature of the branching could easily be overlooked except near the edge of isolated cultures. Further, the regularity of the branching of the delicate protonema would be obscured by the relatively coarse particles of the natural soil or other similar substrata employed by many of the earlier workers.

The heterotrichous habit was observed in all our cultures on solid media, whether on filter paper or agar. At the lower intensity of the artificial light (100 foot-candles) the erect system remained relatively low, as compared with the greater development of this system in natural daylight and in the stronger artificial light (Plate 1, Figs. 7–9). The presence or absence of 1 per cent. glucose, although affecting the pigmentation of the cell-walls, had little effect on the heterotrichous habit. In liquid cultures in the absence of a stable orientation there was naturally no clear distinction between prostrate and erect filaments, but in a number of genera (e.g. *Funaria*, *Pohlia*) there was still a differentiation between branches of limited and of unlimited growth although the cytological differences were less marked than in surface cultures.

It is of interest that one of the early illustrations of the protonema of *Funaria* (Sachs, 1875, Fig. 226) reveals a clear distinction between a dark horizontal filament and erect branches of limited growth. Servettaz (1913) was apparently the first worker to recognize the regular occurrence of aerial filaments. He described two stages in the development of the protonema. During the first ('protonéma rampant') which was said to have a minimum duration of 3 weeks, creeping filaments extended over the substratum, while during the second stage ('protonéma dressé') aerial filaments developed from the former. It is clear, however, that the development does not proceed in two stages, but rather that aerial branches are produced continuously from the prostrate filaments as they spread across the medium. Woesler (1933) recognized the existence of 'aerial' and substrate protonema, and Van Andel (1952) mentioned that vertical aerial branches may be produced, but neither worker appears to have appreciated that the aerial and creeping filaments constitute two distinct systems with differences in cellular characteristics.

The secondary protonema, produced by regeneration from stem or leaf, was not investigated in the present work, but it seems likely from existing accounts, especially from the figures and descriptions of Forest Heald (1898*a*) that this type of protonema will also frequently develop the heterotrichous habit.

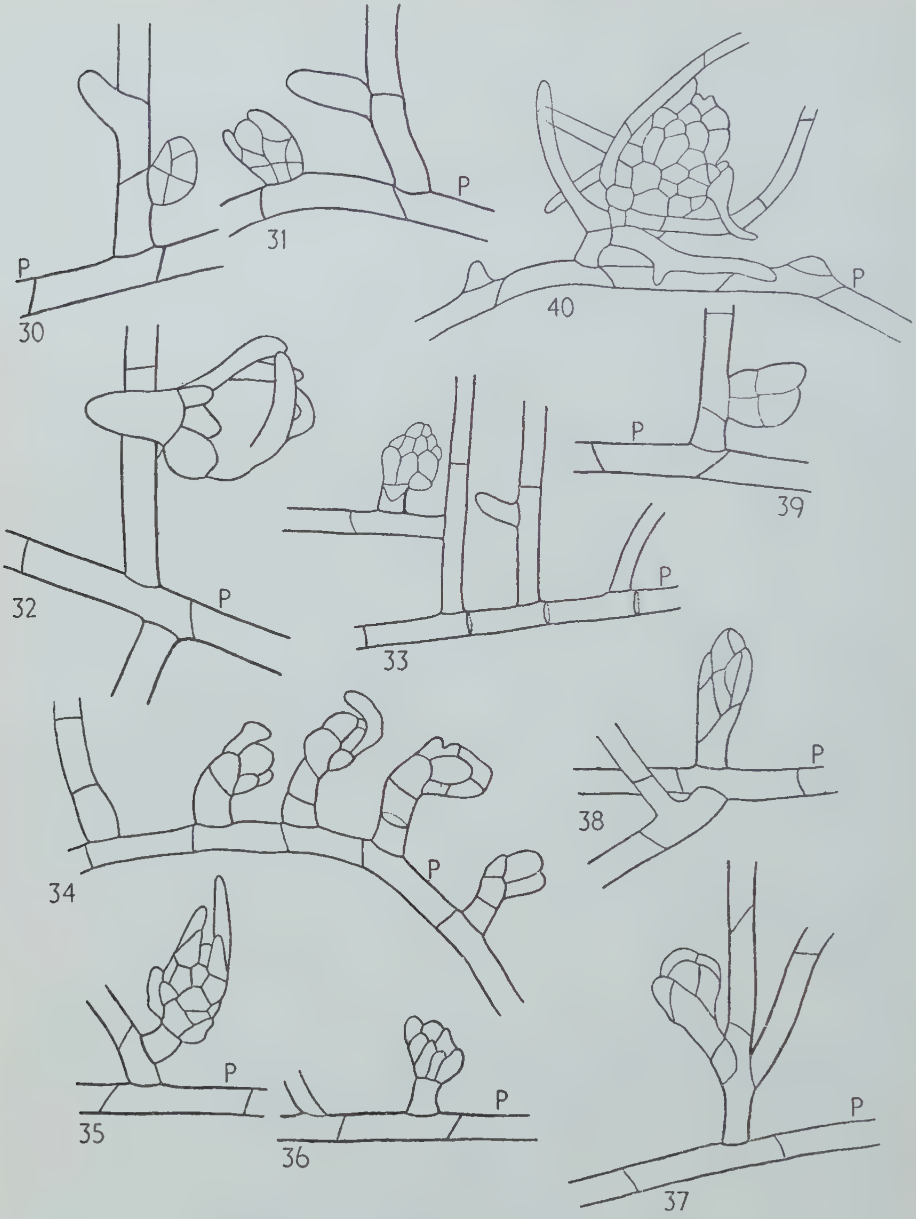
BUD FORMATION

The process of bud formation on the protonema is usually considered to be very uniform throughout the Bryales, but extensive studies in this field have been comparatively few, and it seemed desirable to reinvestigate some of the principal features. Since the studies of Müller (1874) and Sachs (1875) it has been recognized that the buds are most frequently formed near the base of lateral axes of limited growth. Müller also discovered that occasionally buds were produced by the end cell of a main filament. From investigations of a range of species Zederbauer (1902) found that buds, although usually formed from the first cell of a lateral filament, were sometimes also present on the main axes, as in *Ceratodon purpureus*, *Hypnum cupressiforme*, and *Polytrichum commune*, and in one isolated instance on the end cell of a filament.

Most authors (e.g. Müller, 1874; Sachs, 1875; Zederbauer, 1902; Ruhland, 1924; Lorch, 1931; Woesler, 1933; Sironval, 1947) are agreed that the buds arise as a lateral protrusion from a cell of the parent filament which, after cutting off usually one or two stalk-cells, swells at the end and divides by three successive oblique walls to form the three-sided apical cell of the future shoot. It is also known that under certain conditions, particularly in weak light, as many as 20 stalk-cells may be formed (Woesler, 1933). A few workers, particularly Servettaz (1913) and Chalaud (1931), have claimed that bud formation is very variable. Servettaz described the formation of massive buds by segmentation of four terminal quadrants. It is possible, however, that these peculiar structures, provide an example of incomplete differentiation under unfavourable conditions, rather than of a distinct type of bud development.

Our own observations on the formation of buds, although in general agreement with those of previous workers, indicate that there is more variation in position that is usually supposed. The commonest position is near the base of the principal filament of the aerial branch system. This position is characteristic for *Funaria*, *Leptobryum*, and *Eurhynchium*, but even in these genera buds occasionally occurred directly on the brown filaments of the prostrate system (Text-figs. 30–32). In *Mnium*, *Pohlia*, *Ceratodon* and *Pleuridium* buds were found as commonly on the prostrate filaments as on the erect green branches (Text-figs. 33–40). The buds of *Tortula muralis* (Text-fig. 24) were either terminal or occurred laterally on the ultimate branches of the erect system. In *Atrichum* the buds were found in any position on the erect branch system, while in *Bryum* the buds usually occurred near the base of the principal erect filament, but could also be found in any position of the aerial branch system (Text-fig. 23). In all species the buds were usually fairly well spaced, but very rarely occurred on adjacent cells of a main filament (Text-fig. 34). Instances of the formation of more than one bud from a single cell were seen in *Mnium hornum* and *Pohlia nutans*. In *Cinclidotus* the buds were formed all over the rounded protonemal masses (Text-fig. 28).

The segmentation of the buds was not followed in detail, but in all species our general observations were consistent with previous accounts that the development of the bud is due to the activity of a three-sided apical cell, formed by three successive oblique walls in the terminal cell of the short filament from which the bud is usually produced. The surface cells of the bud soon gave rise to protonemal filaments, some of which retained the characteristics of chloronema, while the majority were differentiated as typical rhizoids. Except in *Cinclidotus* the filaments originating from the bud never produced additional buds, evidently as a result of some correlative action exerted by the developing bud. Van Andel (1952), however, described the



TEXT-FIGS. 30–40. Bud formation on protonemata.

Figs. 30, 31. *Funaria hygrometrica.* Fig. 32. *Leptobryum pyriforme.* Figs. 33, 34. *Ceratodon purpureus.* Figs. 35, 36. *Mnium hornum.* Figs. 37, 38. *Pohlia nutans.* Figs. 39, 40. *Pleuridium acuminatum.*

P—prostrate filament. Fig. 39. (×300.) All other figures. (×150.)

formation of buds on chloronema filaments produced by rhizoids growing from the base of moss plants; but since this author failed to find filaments with the characteristics of our 'prostrate system', Sironval's 'caulonema', or Bopp's 'protonema-rhizoids', it seems likely that his bud-bearing rhizoids had not been produced from the moss plants, as he believed, but were really bud-bearing filaments of the primary protonema. In *Cinclidotus*, the dark-brown, exceptionally thick-walled rhizoids gave rise to innumerable buds (Plate 1, Fig. 6; Text-fig. 29). Elssmann (1923) reported the presence of large numbers of buds on the rhizoids of *Cinclidotus* growing under nature conditions. In contrast to our observations in culture, he claimed that the buds arose not from the rhizoids themselves, but from protonema branches of the same. It is also of interest that Paul (1903) concluded from his investigation of the rhizoids of a large number of moss species, that the rhizoids of *Cinclidotus* species were 3–4 times as stout as those of any other genus.

Conditions necessary for bud formation. In the present study no extensive investigation was made of the physiological conditions necessary for bud formation, but the cultural conditions adopted provided a decisive answer to the controversial issues recently raised by Sironval (1947). Sironval claimed that buds were produced only on the 'caulonema' stage of the protonema, which was never attained in pure cultures under artificial light. He considered that the transformation of chloronema into caulonema is a complex phenomenon, which is regulated by two complexes of substances, or at the least by two substances both of which are synthesized in natural light by the chloronema, but one of which is not synthesized in artificial light by the chloronema, although it may be synthesized by certain micro-organisms such as infecting mould-fungi. This hypothesis, which seemed to us to be very unlikely, was definitely refuted by our observation that bud formation in most species occurred as readily under the higher intensity of artificial light (500 foot-candles) as in natural daylight (Plate 1, Figs. 11, 12). Under the weaker artificial light (100 foot-candles) although the growth and differentiation of the cultures were delayed, buds were eventually produced. The formation of buds has even been reported to occur in complete darkness on protonema of species of *Splachnum* growing on a 2 per cent. sucrose medium (Keil, 1949), although most previous workers who were successful in growing protonema in the dark, e.g. E. and O. Pringsheim (1935) failed to obtain the formation of buds. It should also be mentioned that in the present investigation, buds were produced in a number of species (e.g. *Funaria*, *Leptobryum*, and *Cinclidotus*) on relatively undifferentiated filaments growing submerged in the liquid or agar medium. Similar results have been reported by previous workers (e.g. Servettaz, 1913; Meyer, 1940; Keil, 1949).

DISCUSSION

From the present investigation it may be concluded that the protonema of typical representatives of the Bryales attains a higher level of morphological differentiation than has usually been recognized. In particular, the fully developed protonema possesses a markedly heterotrichous habit, with normally a marked distinction between prostrate filaments of relatively unlimited growth and aerial filaments of more restricted development. This completely differentiated condition of the protonema develops only gradually from the more uniform chloronema filaments produced on germination of the spore, but in all our cultures there was no indication of the two distinct stages, 'chloronema' and 'caulonema' identified by Sironval (1947). The characteristics of the 'caulonema', as described by Sironval, are clearly those of the normal, well-grown protonema. They are certainly not associated with a distinct morphological phase, but are probably indicative of a certain nutritional status of the protonema. The process of bud formation, which has already been discussed, is probably also determined by a suitable level of nutrients, and there is no evidence that it is restricted to a separate 'caulonema' phase.

The frequent, and probably almost constant occurrence of a markedly heterotrichous habit in the protonema of the Bryales is also of considerable phylogenetic interest. In 1888, Goebel suggested, on the basis of a comparison between moss protonemata and the sexual generation of certain representatives of the Hymenophyllaceae, that the mosses and pteridophytes originated from algal ancestors composed of branched cellular filaments. More recently Fritsch (1945) put forward a similar hypothesis based on the comparative morphology of existing algae. He was strongly of the opinion that land plants in general evolved from heterotrichous algae closely resembling the Chaetophoraceae of the present day. In his consideration of the Bryophyta, Fritsch wrote that the Musci appear to be the only archegoniates in which both parts of the heterotrichous ancestor have persisted in a single phase, with the protonema representing the prostrate system and the moss plant the erect system.

The recognition that the protonema itself is heterotrichous has now shown that the similarity with the Chaetophoraceae is even greater than Fritsch supposed. Both prostrate and aerial systems occur in the protonema; while the leafy moss shoot is an additional system possibly originally evolved, as suggested by Goebel (1892), for the support and protection of the sexual organs. It is of interest to note that the sexual generation of certain species of the fern genus *Trichomanes* is remarkably similar to a moss protonema. The existing accounts do not enable us to decide whether the filamentous prothallus is characterized by a well-marked heterotrichous habit, but in the genus *Schizaea* (cf. Bower, 1926) the heterotrichous habit is clearly defined. At the present time, the view that the mosses and ferns evolved from heterotrichous algae is little more than a conjecture; but the demonstration of the heterotrichous habit in the moss protonema may be regarded as furnishing additional evidence in favour of those who hold this view.

ACKNOWLEDGEMENTS

We are indebted to Mr. E. Ashby and Mr. G. Barker for the photographs in Plate 1.

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EXPLANATION OF PLATE

PLATE

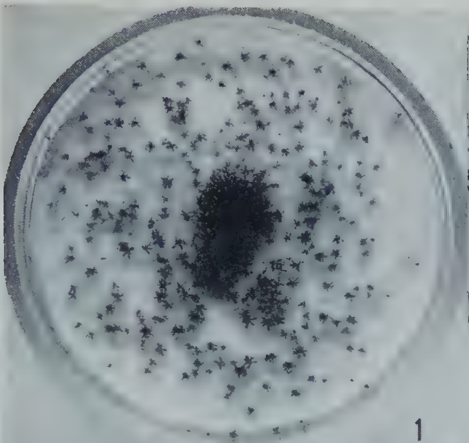
FIGS. 1, 2. *Funaria hygrometrica*: Fig. 1. Culture on mineral agar grown under artificial light (100 foot-candles) showing spiral growth of prostrate filaments of protonema, and production of leafy shoots. (× ⅝.) Fig. 2. Petri-dish culture on mineral agar, grown at 100 foot-candles, showing radial growth of protonema. (× 2.)

FIG. 3. *Ceratodon purpureus*: Petri-dish culture on mineral agar, grown at 500 foot-candles, showing radial growth of protonema. (× 2.)

FIG. 4. *Pleuridium acuminatum*: Part of protonema of culture grown in Petri-dish at 500 foot-candles, showing prostrate and erect (darker) filaments. (× 32.)

FIGS. 5. *Cinclidotus fontinaloides*: Protonema culture after approximately 4 months of growth on mineral agar in test-tube, at 500 foot-candles. (× 4.)

FIG. 6. *C. fontinaloides*: Copious bud formation on stout dark rhizoids, from culture in liquid mineral medium, at 500 foot-candles. (× 20.)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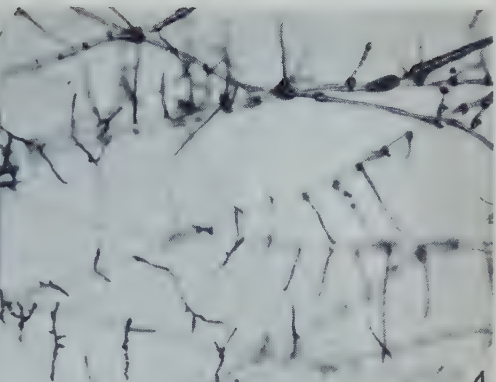
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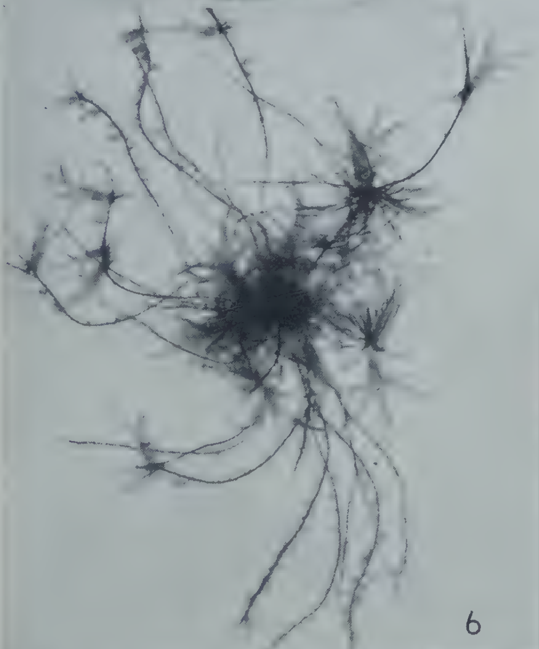
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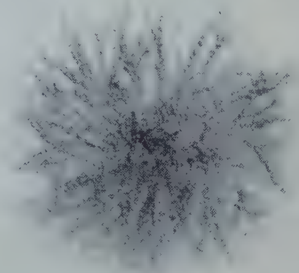
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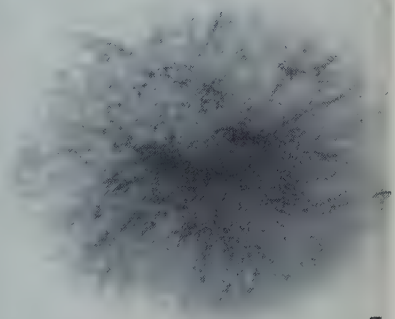
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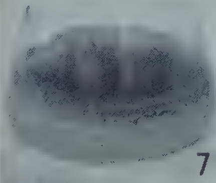
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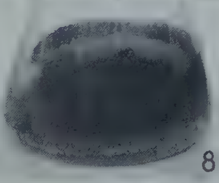
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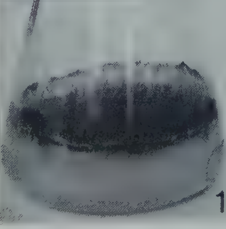
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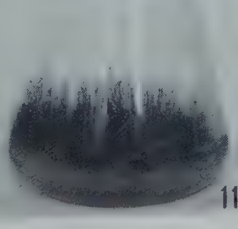
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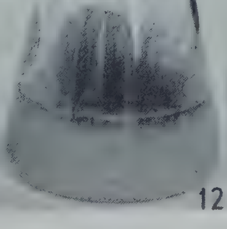
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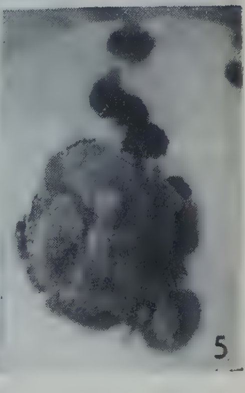
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A. ALLSOPP *and* G. C. MITRA

Plate 1

FIGS. 7–9. *Ceratodon purpureus*: Cultures after 3 months of growth on mineral agar under three different light conditions (Fig. 7, 100 foot-candles, Fig. 8, 500 foot-candles, Fig. 9, natural daylight). Fig. 9 illustrates positive phototropism of tuft of filaments of aerial system. (× ½.)

FIGS. 10–12. *Pohlia nutans*: Cultures after 3 months of growth on mineral agar under three different light conditions (Fig. 10, 100 foot-candles, Fig. 11, 500 foot-candles, Fig. 12, natural daylight) showing (Fig. 11) copious production of leafy shoots in artificial light, and positive phototropism of shoots in natural daylight (Fig. 12). (× ½.)

The effects of Fungi on the Gametophytes of *Pteridium aquilinum* (L.) Kühn

BY

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ABSTRACT

1. The prothalli have been shown to be convenient subjects for the study of some fungal relationships with green plants. Pure cultures have been established from all the populations examined by germination of young spores, and it has been shown that during dormancy the number of spores which can germinate in pure culture decreases more rapidly than the number which can germinate in cultures containing contaminant fungi. Previous reports of the stimulatory effect of fungi on prothallial growth and development have been confirmed and extended.

2. Prothalli growing on mineral agar have been shown to be potentially susceptible to the attack of a wide variety of pathogens of higher plants, but only two naturally occurring species of fungi pathogenic to prothalli have been found during an examination of growth on a variety of soils from bracken-infested areas in the west of Scotland.

3. The aetiology of the disease incited by *Botrytis cinerea* in prothalli has been shown to be similar to that of this disease in higher plants. The pathogen is sometimes restricted to lesions of limited size around the sites of successful infections and this is associated with changes in the cell walls of the host. A protopectinase solution prepared from a culture of germinating spores by Brown's method induced symptoms in the prothallial cells similar to those induced by the attack of the fungus. The cell-attacking properties of the solution were greatly reduced when the protopectinase content was inactivated by low heat for a short time, but they were not entirely destroyed. This confirms previous indications that more than one complex may be involved in the attack, but since the second moiety demonstrated is at least partially thermolabile the evidence does not support judgement of its relative significance in the attacking process.

INTRODUCTION

WORK in this Department during the past seven years has shown that fern gametophytes may be useful tools for the study of some fungal relationships with green plants. The young gametophyte often consists mainly of a layer of almost uniform cells only one cell thick over most of its area. Fungal hyphae growing within the cells can consequently be studied *in vivo* with little disturbance to the conditions of the association, and interpretation of the results is not confused by the presence of areas of specialized tissue. The gametophytes can be grown easily in controlled laboratory conditions, and clonal populations can be built up by vegetative propagation (Mottier, 1927; Hurel-Py, 1950; Wilkie, 1954 and 1956).

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The prothalli of *Pteridium aquilinum* (L.) Kühn have been chosen for initial study because of the amount of previous work which has been done on them (Conway, 1949) and because of the availability of material in this Department. This report deals with the establishment of conditions for their growth in large numbers in controlled conditions and in pure culture, and with a general examination of the effect of common pathogenic fungi on them. A more detailed study of the aetiology of the disease incited by *Botrytis cinerea* has been made, to provide a comparison of the effects of this fungus on the haploid gametophytic green cells of a cryptogam and the diploid sporophytic green cells of a higher plant.

History

The abundant early work reviewed by Mottier (loc. cit.) has shown that a big proportion of spores of many fern species germinate and give rise to normal prothalli on a variety of artificial media. Hurel-Py (loc. cit.) reviewed the previous work on growth in pure culture. This has shown that a smaller proportion of the spores germinate after surface decontamination, and that in some cases abnormalities develop in prothalli grown in pure culture. She found that the growth of prothalli in mineral media was stimulated by the addition of glucose or sucrose and by the presence of contaminant fungi. These authors have also reviewed the early work on vegetative proliferation of prothalli to form colonies of secondary prothalli, and Hurel-Py reported that this occurred only in pure culture. Wilkie (loc. cit.) reported similar findings in *P. aquilinum*. He showed that in one population of 'crested' plants development in pure culture was limited to the formation of a unicellular prothallus, but that the presence of any one of 'a number' of species of fungi and actinomycetes enabled these prothalli to continue development into the adult form. He found that this stimulation was not reproduced by a variety of treatments of spores in pure culture, including the addition of a wide range of vitamins, growth factors, or sterile media from pure cultures of the tested organisms, or by various adjustments to the pH and CO_2 concentration of the cultures. He did not find this phenomenon in two other populations of 'normal' plants which he studied. Conway (loc. cit., 1953) studied spore germination and prothallial growth of *P. aquilinum* in relation to the ecology of the sporophyte, and showed that in naturally contaminated cultures the viability of spores decreased during dormancy.

EXPERIMENTAL

Spores were collected annually from four populations of bracken plants in the west of Scotland, and stored in dry glass tubes at room temperature. Prothalli were obtained by germinating these spores on the surface of the following medium (in Petri-dishes) made up as g. per l. and sterilized at 12 lb. pressure for 30 minutes: KCl, 0·6; $MgSO_4{\cdot}7H_2O$, 0·9; $Ca(No_3)_2{\cdot}4H_2O$, 1·0; KH_2PO_4, 0·6; $NaNo_3$, 0·36; ferric tartrate 0·06; agar agar 20. Spores were decontaminated by washing in 0·1 per cent. aqueous mercuric chloride solution, and all cultures were maintained under 15 hours' illumination daily from fluorescent lights at room temperature. This varied between 16° C. and 26° C.

The observations of Wilkie on the effect of contaminants on prothallial growth were extended by a study of the effect of growing prothalli in association with pure cultures of specific fungi, and the effect of contaminants on spore germination was also examined. To do this Petri-dishes of standard mineral medium were inoculated with the test fungi detailed below and incubated at 24° C. for 48 hours. The fungal colonies were then covered with a sheet of sterile cellophane, and a suspension of decontaminated fern spores which had been collected from Tarbet, Dumbartonshire, eight months previously, was spread over the surface of the cellophane. Control cultures were set up using media without fungal contamination. The resulting germination and growth after 2 weeks in the standard conditions are shown in Table I. The prothalli formed in cultures containing *Pythium debaryanum* were distorted, but those in the other cultures were normal, the effects of the fungi apparently differing only in the degree of stimulation produced.

More detailed studies with *Aspergillus niger* confirmed Wilkie's (loc. cit.) findings that the effect is associated with the presence of the living fungus, and that it is not produced either by sterilized filtrates from liquid cultures of the fungus, or by sterilized agar on which fungi have grown. Naturally contaminated cultures of untreated spores were observed to show similar effects, while the number of spores which could germinate in pure culture was found to decrease during dormancy more rapidly than the number which could germinate in cultures containing contaminant fungi.

TABLE I

The Effect of Pure Cultures of Fungi on Spore Germination and Prothallial Growth of Pteridium aquilinum *(L.) Kühn*

Fungus present	*Mean* % germ n.	Usual size of prothalli
None	59	one cell
Pythium debaryanum Hesse CMI 48558	71	2–3 cells
Penicillium notatum West. Departmental culture	74	3–5 cells
Botrytis cinerea Pers. Departmental culture	79	4–6 cells
Aspergillus niger Van Teigh. CMI 17454	85	7–12 cells
Mixed fungal contaminants in culture of untreated spores	81	8–14 cells

Each record is based on a count of 600–750 spores distributed over four replicate Petri-dishes.

THE PATHOLOGY OF BRACKEN PROTHALLI

The delicate structure and humid normal habitats of prothalli appear to give suitable conditions for pathogenic attack, though there have been few studies of their pathology. Mottier (loc. cit.) reported that fungi 'gave little trouble' in cultures of *Matteuca nodulosa* and *Osmunda claytoniana,* only one small culture being lost through fungal attack during 4 years' study. Gregor (1938) summarized earlier reports which indicate that various prothalli are susceptible to the attack of a number of damping-off fungi including *Cephalothecium roseum* and *Pythium* spp., and to that of a specialized parasite *Completoria complens* Lohde. Hurel-Py (loc. cit.) found that prothalli grown in pure culture showed little resistance to the attack of many fungi and bacteria, and Conway (loc. cit.) reported a disease of bracken prothalli which was shown to be incited by a new species of *Tilachlidium* (Hutchinson, 1953). This meagre and unrelated information gives little idea of the potential susceptibility of prothalli to disease, and a preliminary survey was therefore carried out in two phases, viz.:

(*a*) A survey of the effects of common pathogens of higher plants.

(*b*) The isolation, identification and study of pathogens found as contaminants in culture.

(*a*) *Survey of the effects of common pathogens of higher plants on prothalli*

Five equal sized prothalli, aged 5 weeks, were placed in each of a series of Petri-dish cultures, and after 2–3 days' further incubation groups of these dishes were inoculated with pathogenic fungi. An arbitrary range of pathogens of higher plants was chosen from those readily available so as to include species with a wide host range, species inciting a variety of types of disease, and representatives of the main classes of fungi. Their effect was studied by placing a small piece of an agar culture of the test fungus against the edge of the plant, and recording disease development daily.

All the parasites which infected the prothalli incited the same type of symptoms, the difference between them being only in the rate and degree of development of disease. The first visible reaction in the host was in most cases the formation of brown pigment in the walls immediately around the sites of initial penetration by the fungus. The contents of the cells penetrated by the fungus later became disorganized, the chloroplasts usually disappearing early in the attack. Subsequently the whole of the walls of the attacked cells often became brown, but they remained colourless in some cases, particularly in those in which the disease progressed very rapidly. Later the cell contents disintegrated completely, the cell walls fell apart and the whole infected area became a disorganized mass of fragments permeated by fungal hyphae.

In some individuals of each batch of prothalli the pathogens caused initial infections but did not spread generally through the host tissues. In these plants the attacked area was normally surrounded by a continuous line of dark-brown cell walls which the fungal hyphae could not penetrate.

More detailed examination of the infection by *Botrytis cinerea* showed that the formation of these localized lesions was not consistently associated with any obviously characteristic cultural conditions, and that they frequently developed in only one of several distinct sites of infection in plants which had received multiple inoculae. Reinoculation of the plants at other sites with mycelium from fresh cultures, or from the colony in the lesion, resulted in infections, and hyphae from these frequently grew through the host cells until they came into direct contact with the brown wall around the original lesion. There was no sign in any experiment of any inhibition of fungal growth within the cytoplasm of the cell cavities.

The plants were examined 10 days after inoculation, and placed in one of the following classes by empirical observation:

(i) Generally diseased, the fungus spreading freely through the host.
(ii) Locally diseased, the fungus being confined to a local area around the site of initial infection.
(iii) Uninfected.

The results are recorded in Table II.

TABLE II

The Effect of Common Pathogens of Higher Plants on Prothalli

Fungi	Number of plants tested	Number of plants which were: (i) Generally diseased	(ii) Locally diseased	(iii) Healthy	Area of infection as per cent. of total area of prothalli†
Phytophthora parasitica	60	19	19	22	40
Rhizopus nigricans	60	12	12	36	20
*Fusarium solani**	40	0	0	40	0
Fusarium caeruleum	60	8	4	48	20
Helminthosporium sativum	59	49	6	4	70
Aspergillus niger	58	0	16	42	10
Botrytis cinerea	110	95	25	0	85
Botrytis allii	60	37	6	17	60
Trichothecium roseum	60	3	15	42	10
Corticium solani	40	3	13	24	10
Pythium debaryanum	100	100	0	0	100
Cephalothecium roseum	70	69	1	0	90
Fusarium vasinfectum	82	9	15	57	10
*Verticillium dahliae**	60	4	4	42	10
Verticillium albo-atrum	90	0	13	77	7
Sclerotinia sclerotiorum	40	27	9	4	70
Phoma betae	40	0	0	40	0
Rhizoctonia solani	60	43	7	10	70
Tilachlidium nigrum	89	54	25	10	60
Alternaria brassicae	56	0	7	49	5

* The virulence of these strains of *F. solani* and *V. dahliae* was tested at the time of this experiment by inoculating cress seedlings growing in pure culture on mineral agar with similar inoculae to those used on the prothalli. They were both highly virulent, causing typical symptoms within a few days of inoculation.

† The area of infection was estimated by eye as a percentage of the total surface area of the prothallus, the observations of both authors being consistent.

(*b*) *The isolation and study of pathogens found as contaminants in culture*

During the course of this work many thousands of prothalli have been grown on standard media from untreated spores from various sources. Isolations were made of all fungi found on diseased prothalli, and the isolates were tested for pathogenicity by inoculation on to prothalli in pure culture. It was realized that this process limited the possibility of study to the bracken-spore-borne and to airborne parasites, and further experiments to examine the possible occurrence of soil-borne parasites were set up by planting decontaminated spores on to the surface of samples of soil from a wide variety of bracken-infested localities in the west of Scotland. The planted samples were kept in damp and warm conditions and isolations made from any diseased prothalli which appeared. The common contaminants of cultures both on standard agar and on soil, inoculated either with decontaminated or with non-treated spores, were species of *Penicillium*, *Aspergillus*, *Trichoderma*, and bacteria. These rarely attacked the prothalli directly, though in favourable conditions for their growth they occasionally smothered slowly growing young prothalli. Only two actively parasitic species were isolated from the many hundreds of cultures which were examined. These were a new species of *Tilachlidium*, which was reported previously (Hutchinson, loc. cit.), and a new species of *Coniothyrium*. These both caused typical symptoms on inoculation into pure prothallial cultures, and a detailed account of them will be given elsewhere.

THE AETIOLOGY OF BOTRYTIS DISEASE OF PROTHALLI

A more detailed study was made of the effects produced by *Botrytis cinerea*. This has shown that its methods of attack and effects on the host are similar to those on higher plants. The formation of apressoria and infection pegs has been followed *in vivo*, and the mechanical effects of pressure exerted by the living hyphae have been seen in the invagination of the host cell walls and the bending of the hyphae as they come into contact. The addition of nutrients to the infection drop caused an increase of infection, and variation of the concentration of spores in the inoculum on either side of a defined optimum caused a reduction in infection. The detailed experimental evidence for these findings is not presented as they merely confirm those of other workers who studied *Botrytis* disease in higher plants (Brown, 1915–55). The effect of protopectinase secreted by the fungus was studied by Brown's method (1915) as modified by Wood (1953). The spores from a 10-day-old Petri-dish culture of *B. cinerea* on potato agar were scraped into 10 ml. of glucose-asparagine medium (glucose 20 g., asparagine 2·5 g., KH_2PO_4 1·0 g., $MgSO_4 \cdot 7H_2O$ 0·3 g., distilled water 1,000 ml.), the suspension was filtered through muslin, and 5 ml. aliquots used to inoculate each of a series of 35 ml. aliquots of the same medium contained in 250 ml. conical flasks. After 5 days' incubation in the dark at 20° C. the medium was removed by filtration through muslin and centrifuged for 5 minutes to remove the residual spores and mycelium. The effect of this filtrate was tested by injecting prothalli with water under vacuum, draining them quickly on filter paper, immersing them in the filtrate and examining them microscopically at regular intervals. In each of five replicate experiments the contents of the cells in scattered areas of the prothalli became visibly disorganized within 10 to 30 minutes of immersion, the chloroplasts scattering to leave a peripheral colourless area of the cytoplasm, and the cell walls eventually appearing as a thin contorted line. Within 3 hours the numbers of damaged cells increased rapidly, and within 4–6 hours all cells of the prothalli were visibly affected. In this stage they fell apart immediately on handling. The chloroplasts remained green for several hours after the cells were disorganized, and no brown colour developed in the cell walls at any time in prothalli which were completely immersed in the filtrate. Drops of the filtrate incited similar symptoms in the underlying cells when placed on the surface of healthy prothalli, except that some of the cell walls became brown, particularly around the edge of the drop. The effect of heat on the activity of the filtrate was examined by heating and testing samples as shown in Table III. The protopectinase activity of the treated samples was tested by Brown's potato disc technique. The results in the table show that the samples heated *for short periods* no longer possessed the power to macerate potato discs sufficiently to be detected by the test, but that they still retained some activity against the prothallial cells. To determine whether this effect on the prothallial cells was due to the presence of a residual concentration of unaltered protopectinase which was too low for detection in the potato disc test, samples of the original filtrate were diluted with unfermented glucose-asparagine medium as shown in Table IV, and tested in the same way. The results shown in Table IV indicate that a concentration of protopectinase which had no apparent effect on the prothallial cells had a perceptible effect on potato discs.

Microscopic examination of the prothalli used in experiments summarized in Tables III and IV showed that heated fungal filtrates did not cause appreciable maceration of the cell walls of the prothalli in any experiment. On the other hand it was seen that cells of prothalli soaked in the untreated filtrates separated along their middle lamellae on pulling apart, while those soaked in unfermented glucose-asparagine medium or in any of the heated filtrates separated by breaking irregularly across their walls and cytoplasm.

Discussion

This work was carried out to obtain the information needed to plan some detailed investigations of host reaction to parasitism which are now in progress. It has shown that stocks of gametophytes can be established conveniently in pure culture on mineral media by germination of young spores. The reduction during dormancy of ability to germinate is similar to that commonly caused by the breakdown of a store of essential metabolite or metabolites contained in the young spore, or to the loss of ability to synthesize this material during germination. The phenomenon is distinct from that of the loss of ability for completely autotrophic development of germinated spores into normal adult prothalli, and within a limited storage time this loss can apparently be counteracted by the action of some secretion from the living hyphae of a variety of fungi. The nature of this stimulatory action has not been examined, but the fungi tested stimulated development of prothalli under the same conditions as they stimulated spore germination, and it may well be that the same materials are involved in each phenomenon. These losses of metabolic ability are confined either to dormancy or to the early stages of germination, as prothalli which had established themselves autotrophically can be maintained in pure culture for many generations by

TABLE III

The Effect of Heating on the Activity of Filtrates from Cultures of B. cinerea

	Treatment of filtrate						
	Unfermented Glucose-Asparagine medium	Untreated filtrate from cultures	Filtrate heated to 65° C. for 1 min.	Filtrate heated to 65° C. for 5 min.	Filtrate heated to 98° C. for 1 min.	Filtrate heated to 98° C. for 5 min.	Filtrate heated to 120° C. for 10 min.
Proportion of disorganized prothallial cells after 24 hrs. immersion in filtrate	0%	90%–100%	15%–25%	5%–15%	10%–15%	0%–2%	0%
Appearance of prothallial cell walls after 24 hrs. immersion in filtrate	Unchanged	Macerated*	Unchanged	Unchanged	Unchanged	Unchanged	Unchanged
Appearance of potato discs after 24 hrs. immersion in filtrate	Unchanged	Macerated†	Unchanged	Unchanged	Unchanged	Unchanged	Unchanged

* Maceration was evident after 4–6 hours. † Maceration was evident after 20–40 minutes.

The results are a summary of four experiments in each of which four prothalli or potato discs were examined in each sample. The proportion of disorganized cells was estimated by eye as in Table II.

TABLE IV

The Effect of Dilution on the Activity of Filtrates from Cultures of B. cinerea

	Dilution of filtrate									
	Original filtrates	1/10	1/15	1/20	1/25	1/30	1/40	1/50	1/75	1/100
Time taken to macerate potato discs	25–40 min.	1½–2½ hrs.	2–2½ hrs.	2–3 hrs.	2½–3½ hrs.	3–4 hrs.	3½–4½ hrs.	5–7 hrs.	7–9 hrs.	12–24 hrs.
Appearance of prothallial cell walls after 24 hrs. immersion	Macerated	Macerated	Macerated	Macerated	Macerated	Macerated	Unchanged	Unchanged	Unchanged	Unchanged
Proportion of disorganized cells in prothalli after 24 hrs. immersion	100%	100%	80%–100%	80%	60%	50%–60%	20%–40%	5%–20%	0%	0%

The results are a summary of two experiments in each of which four prothalli in discs were examined in each sample. The time of maceration recorded was that from first immersion in the liquid until the first appreciable reduction in resistance to being pulled apart by hand, and the proportions of disorganized prothallial cells were estimated as in Table II.

vegetative propagation without loss of vigour. The phenomena were seen in the spores of all the populations which were examined, and while more work is required to determine their precise distribution and significance it is possible that Wilkie's finding of loss of ability for autotrophic growth in only one of the three populations which he examined may have been associated with differences in age of the spores used.

The examination of the effects of pathogens of higher plants was designed only to give an indication of the general susceptibility of the prothalli to fungal diseases. The results show that the prothalli are susceptible to the attack of a large number of common fungi in the conditions employed. Some common vascular wilt-inciting fungi did not attack them in these experiments, but as virulence against normal hosts was tested in two cases only no valid conclusions can be drawn from failures of infection in these or in other fungi which were examined. This susceptibility seems to add much to their problems of survival in the humid conditions of their normal habitats, and it makes it difficult to understand the infrequent occurrence of diseased prothalli in the untreated soil cultures.

The more detailed study of *Botrytis* disease was undertaken to see whether there are any major differences between the aetiology of this disease on prothalli and on higher plants. The results show that there is at least a very close similarity between the two, though of course the properties of green cells of such widely differing plant classes should be compared with caution. The observations of the living hyphae of this fungus as they penetrate and grow within the cells of the host give striking confirmation of the classical theory of penetration by mechanical pressure. They also showed clearly that the containment of infections in localized lesions is due to changes in the cell walls, as hyphae were seen growing actively within the cytoplasm of cells on either side of the limiting brown cell wall but were never seen to penetrate it. The application of Brown's techniques to this plant has confirmed that much of the anticellular activity is associated with the presence of protopectinase in the complex of metabolites secreted by the fungus, and Tribe's finding (1955) that some moiety of this metabolite complex is still active against host cells after the inactivation of the protopectinase by low heat. The dilution experiments show that in this case this moiety is not a small residue of undestroyed protopectinase. The effect of continued heating of the fungal filtrates shows that this active moiety is not completely heat stable, however, and there is therefore insufficient evidence on which to judge the relative contribution it makes to the total activity of the untreated culture filtrates.

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Lead Hydroxide as a Selective Absorbent for Certain Organic Constituents of Plants

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ABSTRACT

Freshly precipitated lead hydroxide has been used for the chromatographic analysis of plant extracts. Brilliantly coloured bands were produced with a wide range of colour, e.g. purple, green, yellow, and white, which were characteristic of the variety. A lead hydroxide suspension could also be 'titrated' with the plant extract to a sharp end-point, and the original constituent could be recovered in a relatively pure state from the centrifuged solid by the addition of sulphuric acid.

INTRODUCTION

EXTRACTS of the plant material in aqueous methanol were obtained in the apparatus described elsewhere (Roach, 1957). Removal of the alcohol precipitated the chlorophyll, carotenoids, and most of the phloridzin, and the clear aqueous supernatant liquid was chromatographed. A suspension of freshly precipitated lead hydroxide was used both in chromatographic columns and as a means of separating certain plant constituents, e.g. an anthocyanin, in a relatively pure state (Roach, 1932).

This preliminary work was not extended and when later the organic constituents of plants were explored systematically (Bradfield and Flood, 1950), advantage was taken of the methods of partition chromatography which by then had become well established. A brief description of the lead hydroxide methods is given as they may still be useful in certain cases.

PREPARATION OF LEAD HYDROXIDE SUSPENSIONS

Two solutions are required; (*a*) lead nitrate solution, 100 g./litre and (*b*) ammonia solution, 40 ml. 0·880 ammonia/litre. Both solutions were brought to 63° C., poured at the same rate into a large beaker and then stirred. The precipitate was allowed to settle and most of the supernatant liquid decanted, leaving a lead hydroxide suspension.

PREPARATION OF LEAD HYDROXIDE COLUMNS

The above suspension was poured into a glass tube of the usual form provided with a plug of cotton wool as a filter. The resulting column 20 cm. × 2·5 cm. (diam.) was washed with water at a rate of 2 ml./min. until the effluent gave no positive test for nitrate ion.

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CHROMATOGRAPHY OF PLANT EXTRACTS

Plant extract was added carefully just before the level of the washing water reached the top of the lead hydroxide and the column developed with water or methyl alcohol. Brilliantly coloured bands were formed and these differed markedly according to the variety. Thus Eaton (1950) recorded that the colour of the bands from top to bottom for Malling rootstock M. IV were: Naples yellow, aniline yellow, citrine, citron yellow, naphthalene yellow, and for the variety Allington Pippin they were: light yellow, yellow ochre, laurel green, pale green, pale yellow.

Eaton also noted a quantitative difference within the same variety according to whether it received applications of potassic manure or not.

SEPARATION OF PURE SUBSTANCES

Preliminary separations could obviously be made by extruding the columns, separating the variously coloured bands, and recovering the original substances by the addition of dilute sulphuric acid.

An alternative, and for some purposes a better, method consisted in doing a rough 'titration' by adding the lead hydroxide cream to the extract using a colour change as an indicator and doing a sharper back 'titration' with the sulphuric acid. Thus an anthocyanin was isolated from Allington Pippin extract by adding the lead hydroxide until it was coloured faintly green. The mixture was centrifuged and the red solution poured off. To this, lead hydroxide was added until the supernatant solution was no longer red. The mixture was centrifuged, and the supernatant liquid poured off. The green lead salt was triturated with dilute sulphuric acid, using the disappearance of all green colour in the precipitate as end point.

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